

CROSS-MODULATORY ACTIONS OF CELL CYCLE MACHINERY ON  
ESTROGEN RECEPTOR- $\alpha$  LEVEL AND TRANSCRIPTIONAL  
ACTIVITY IN BREAST CANCER CELLS

BY

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DISSERTATION

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## THESIS ABSTRACT

Breast cancer is one of the most highly diagnosed cancers in women and the second largest cause of death of women in United States. The anti-estrogen tamoxifen which blocks gene expression through estradiol bound ER $\alpha$ , and hence the growth stimulatory effects of estradiol, has been widely used for decades for treating patients with ER $\alpha$  positive or hormone dependent breast cancer. Despite its obvious benefits, in as high as 40% of the patients receiving tamoxifen therapy there is an eventual relapse of the disease largely due to acquired resistance to the drug, underlying mechanism for which is rather poorly understood. Elucidating the molecular basis underlying “acquired tamoxifen resistance” and agonistic effects of tamoxifen on cellular growth was the primary focus of my doctoral research. We addressed this by two approaches, one being studying the molecular mechanism for the regulation of cellular levels of ER $\alpha$  so as to prevent its loss in ER $\alpha$  positive or restore its levels in ER $\alpha$  negative breast cancers and second to investigate the role of tamoxifen in modulating the expression of ER $\alpha$  target genes independent of estradiol as a function of its stimulatory or estrogenic effects on breast cancer cell growth.

To this end we found a novel mechanism involving the E-3 ubiquitin ligase and major regulator of cell cycle progression, Skp2, in the proteasomal degradation of ER $\alpha$  upon activation by p38MAPK. Interestingly, this regulation of ER $\alpha$  by sequential actions of p38MAPK and Skp2 that was observed specifically during the G1/S transition and S-phases of the cells cycle was found to be critical for the progression of cells through S-phase. The underlying mechanism of increased cell cycle entry due to concerted actions of ER $\alpha$  and Skp2 was later identified to be due to positive regulation of the trans-activation function of ER $\alpha$  by Skp2, most likely due to efficient cycling of the receptor on target gene promoter upon Skp2 driven turnover. The impact of Skp2 on ER $\alpha$  mediated growth of tamoxifen

resistant breast cancer cells was far more pronounced in the presence of tamoxifen than estradiol. In order to find the likely mechanism driving Skp2 mediated growth through ER $\alpha$ , in the presence of tamoxifen, we attempted to identify co-regulators unique to tamoxifen bound ER $\alpha$  and identified Oct-3/4, a transcription factor with well-known role in embryonic stem cell growth and differentiation, to be a specific regulator of tamoxifen, not estradiol, mediated gene regulation through ER $\alpha$ . Our work highlights a novel mechanism for the induction of Oct-3/4 in response to tamoxifen, not estradiol, largely due to p38MAPK initiated and Skp2 mediated degradation of Nkx3-1, a co-repressor of Oct-3/4 expression through ER $\alpha$ . Interestingly, further investigation revealed Oct-3/4 transcription factor binding sites were enriched in genes that recruited ER $\alpha$  preferentially or exclusively in the presence of tamoxifen (by >5 fold compared to estradiol). Oct-3/4 induction by tamoxifen appeared to be followed by recruitment of Oct-3/4 to a subset of genes that were targets of tamoxifen bound ER $\alpha$ , a phenomenon that facilitated the recruitment of ER $\alpha$  to its binding sites in close proximity. One such candidate target, NFATC4, was shown to be induced by ER $\alpha$ -Oct-3/4 axis through involvement of Skp2 and p38MAPK, in the presence of tamoxifen, not estradiol. Interestingly, Oct-3/4 was found to significantly impact on tamoxifen driven growth of breast cancer cells, largely through its actions on ER $\alpha$  target gene expression in response to tamoxifen. Consistent with this, Oct-3/4 protein expression was much elevated in MCF-7-Tam<sup>r</sup> cells (tamoxifen resistant derivatives) compared to parental MCF-7 cells.

Taken together, these findings reveal a unique mechanism underlying the regulation of cellular levels of ER $\alpha$  in breast cancer cells as well as the specific role of tamoxifen in regulating ER $\alpha$  target gene expression independent of estradiol thereby promoting growth of breast cancer cells. These pathways we believe offer new targets for drug discovery to circumvent tamoxifen resistance in breast cancer thereby making tam-resistant tumors amenable to therapy.

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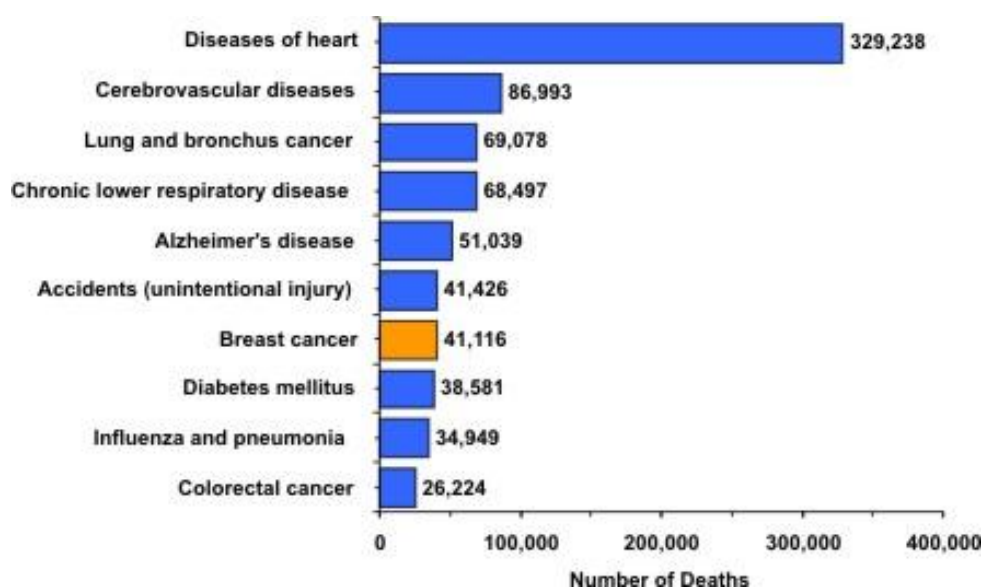
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# CHAPTER I-ESTROGEN RECEPTOR SIGNALING AND BREAST CANCER

## INTRODUCTION

### *Breast Cancer Occurrence and Associated Mortality*

Breast cancer is the most common cancer amongst women, and the second leading cause of cancer death, exceeded only by lung cancer. Women in the United States have the highest incidence rates of breast cancer in the world with a 1 in 8 (12.5%) lifetime chance of developing invasive breast cancer and a 1 in 35 (3%) chance of breast cancer causing their death.



***Fig.1.1 Top 10 Causes of Death for Women in the United States†***

The 5-year survival rate refers to the percentage of patients who live at least 5 years after being diagnosed with cancer. †Source: Surveillance, Epidemiology, and End Results (SEER)

### ***Estrogen Receptor: Target for Breast Cancer Therapy***

Estrogen receptor  $\alpha$  (ER $\alpha$ ), a nuclear hormone receptor, is a master transcription regulator of genes involved in controlling growth and proliferation of the breast and hence the main target of

endocrine therapies [1, 2]. Approximately 70% of human breast tumors depend on estrogens (the naturally produced cognate ligand for ER $\alpha$ ) for proliferation, making them amenable to treatment with drugs such as anti-estrogens (tamoxifen) also known as selective estrogen receptor modulators (SERMs) and/or aromatase inhibitors (letrozole), which block the signaling by ER $\alpha$ . Tamoxifen has been used for the systemic treatment of patients with breast cancer for nearly three decades [3]. Tamoxifen is a selective ER modulator with mixed ER agonist/antagonist activities that is thought to work by competitive blockade of estrogen mediated ER activity by competing with E2 for LBD binding, inducing a conformational change distinct from E2-ER $\alpha$  complex, leading to inactivation of the AF-2 domain and receptor stabilization. When tamoxifen is bound to the ER it also interacts with co-repressors resulting in suppression of E2 mediated agonistic activity. Since the effects of tamoxifen are primarily mediated through the ER, the degree of ER expression is a strong predictor of response to tamoxifen therapy and its loss could confer resistance [4-6]. Although anti-estrogens, particularly tamoxifen, have proven to be effective in the treatment of breast cancer, they show effectiveness only against ER-positive tumors while the ER-negative tumors fail to respond [7], [8], [9-12], [13], [14, 15], [16-18], [19]. Even amongst ER-positive tumors, about 40% fail to initially respond to tamoxifen therapy, and of those breast tumors initially responding many eventually develop resistance thereby showing a poor survival and greater recurrence rate.

The possible causes for intrinsic and acquired resistance have been attributed to the pharmacology of tamoxifen, alterations in the structure and function of the ER, changes in the expression of ER $\alpha$  or ER $\beta$  (the two major subtypes of the estrogen receptor), alterations in co-regulatory proteins, the influences of growth hormone receptor initiated signal transduction pathways, the interactions with the tumor environment and genetic alterations in the tumor cells. However, no prominent mechanism leading to resistance has been identified so far. A better understanding of the



molecular basis of anti-estrogen resistance therefore could help in developing new therapeutic strategies for breast cancer treatment that may circumvent hormone resistance.

### ***Estrogen Receptor: Structure, Function and Isoforms***

Estrogen Receptor, a member of the steroid hormone superfamily, is a ligand induced transcription factor that has a complex structure containing various functional domains namely, two activation function domains (AF1 and AF2), a DNA binding domain (DBD), a hinge region and a ligand binding domain (LBD). The N-terminal A/B domain of the receptor contains the AF1 domain which is capable of constitutive transcriptional activation in the absence of hormone. The DBD that is located towards the central part of the receptor functions to directly bind to estrogen response elements (EREs) that helps the recruitment of the receptor to the promoters of its target genes. The DBD contains two non-equivalent zinc fingers. The amino terminal zinc finger is responsible for the recognition and direct interaction with the ERE and the carboxy terminal zinc finger is responsible for the stabilization of ER bound to the DNA through the phosphate backbone of the DNA double helix. The hinge region adjacent to the DBD contains the nuclear localization sequence and has been shown to be necessary for its interaction with the heat-shock proteins. ER also contains a carboxy terminal ligand binding domain (LBD) that is required for the interaction of the receptor with various estrogenic ligands. This domain that contains the AF2 experiences a conformational change upon ligand binding that facilitates co-regulator recruitment. AF2 domain activity is influenced by post translational modifications induced by various cellular kinases such as MAPK and AKT [17, 20, 21], [22].

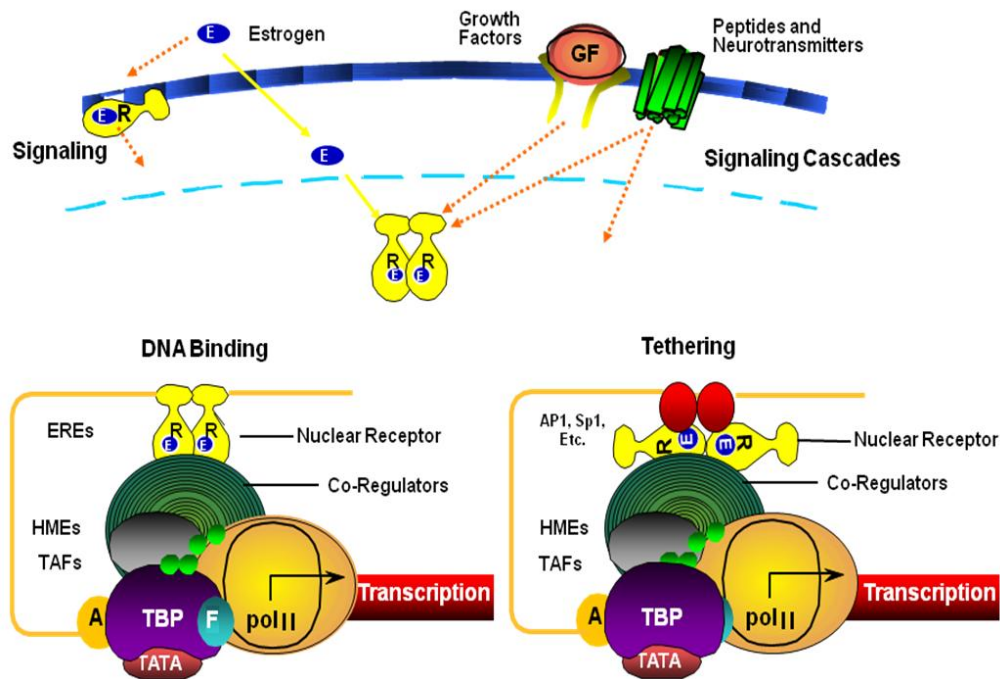
ER $\alpha$  which was discovered in 1986 was the only ER isoform known until 1995, when almost a decade later another ER isoform called ER $\beta$  was identified. Since then, much effort has gone into identifying specific expression patterns, functions and possible cross-modulatory effects of the two

isoforms on each other. Although both receptors co-express in various reproductive and non-reproductive tissues, ER $\alpha$  is the predominant isoform in uterus, liver, heart and kidney while ER $\beta$  expresses at much higher level in lung, ovary, prostate, and central nervous and the hematopoietic systems. Most studies till date identify ER $\beta$  to be a negative regulator of ER $\alpha$  actions of promoting cellular growth and proliferation. Both ER $\alpha$  and ER $\beta$  share the same structural domains, with some domains having high degree of sequence homology while others totally non-equivalent. The two ER isoforms share the highest sequence homology in the DBD (97%), followed by the LBD (57%), the hinge region (30%) and finally the AF1 (18%) and the tail domain (18%). It is believed that because of the high homology in the DNA binding domain, the two receptors may compete for the same binding site thereby exerting a cross-modulatory effect on each other's actions while still performing unique cellular functions by recruiting different co-regulator proteins to target gene promoters owing to the divergent AF1 domain. This highlights the unique modes of gene regulation through the two ERs despite the fair amount of homology in the overall protein sequence [23].

### ***Estrogen Receptor: Modes of Action and Gene Regulation***

Estrogen Receptor  $\alpha$  is a master transcription regulator that mediates the biological effects of estrogens in a variety of target tissues thereby regulating cellular growth and differentiation. In its classical mode of action it binds the naturally produced hormone, Estrogen (E2), which acts as a cognate ligand for the receptor, translocates into the nucleus followed by dimerization and direct binding/docking to DNA sequences containing specific response elements, GGTCAnnnTGACC, called EREs (Estrogen Response Elements) in the promoters of target genes. The binding of E2 to the ligand binding domain (LBD) of ER $\alpha$  also induces a conformational change of the receptor facilitating the recruitment of co-regulatory proteins and basic transcription machinery impacting on the expression of

target gene(s), based on cell and promoter context, followed eventually by rapid ER $\alpha$  degradation. However, about one third of human genes regulated by ER $\alpha$  lack ERE-like sequences (Hansen U 2004). These genes are largely regulated through the non-classical mode of ER $\alpha$  action whereby instead of direct DNA binding, the receptor is recruited to the promoters of target genes by tethering to a now growing list of transcription factors such as AP-1, SP-1, NF $\kappa$ B, PITX 1. As seen with the classical genomic action of ER, the non-classical ERE-independent genomic action then recruits factors, co-regulatory proteins and basic transcription machinery, which decides the transcriptional outcome of the target genes [24-26], [27-29], [30-32].



Model Adapted from B. Katzenellenbogen et al. Recent Prog. Horm. Res., 2000

### ***Fig.1.2 Modes of Action and Gene Regulation for Estrogen Receptor***

Estrogen Receptor  $\alpha$  is a master transcription regulator. In its classical mode of action it binds Estrogen (E2) and translocates into the nucleus followed by dimerization and direct binding/docking to DNA sequences containing specific response elements, GGTCAnnnTGACC, called EREs (Estrogen Response Elements) in the promoters of target genes. In the non-classical mode of ER $\alpha$  action instead of direct

DNA binding, the receptor is recruited to the promoters of target genes by tethering to a now growing list of transcription factors such as AP-1, SP-1, NF $\kappa$ B, PITX 1.

### ***Estrogen Receptor: Ligands and Co-regulator Recruitment***

There is a range of both naturally produced and chemically synthesized ligands for ER identified till date. These ligands fall into two broad categories based on functional specificity towards ER isoforms such as, full agonist (17 $\beta$ -estradiol), partial agonist/partial antagonist (tamoxifen, raloxifene), full antagonist (ICI 182, 780) and ER $\alpha$  selective agonist (PPT), ER $\beta$  selective agonist (DPN). The character of these ligands is cell-type, tissue and promoter selective and depends on the expression of ER co-regulators. For instance, 4-hydroxy tamoxifen acts as an antagonist to ER mediated target gene expression and proliferation in the breast, while exerting estrogenic properties in the uterus. According to previous studies, this tissue selectivity of TOT can be attributed to the higher level of co-activator protein SRC-1 in the uterus than in the breast. Raloxifene, on the other hand, exerts estrogenic properties in maintaining bone density while being anti-estrogenic in both breast and uterine tissues. Based on their tissue selective activities, tamoxifen and raloxifene are now more appropriately referred to as SERMs (Selective Estrogen Receptor Modulators).

Liganded steroid receptors regulate gene expression by associating with and recruiting coregulatory proteins to gene promoters by association through their activation function domains. These co-regulators can either function as co-activators and enhance steroid hormone receptor driven target gene expression or act as co-repressors and dampen it [33, 34], [6, 35, 36], [32, 37, 38], [39-41]. Co-regulators are often classified into two broad functional categories, covalent histone modifiers and ATP dependent chromatin remodelers [42-44]. They possess intrinsic enzymatic activity by virtue of which they make modifications to histones that include acetylation, methylation, phosphorylation, ubiquitination and sumoylation [45, 46]. These modifications alter the accessibility of members of the

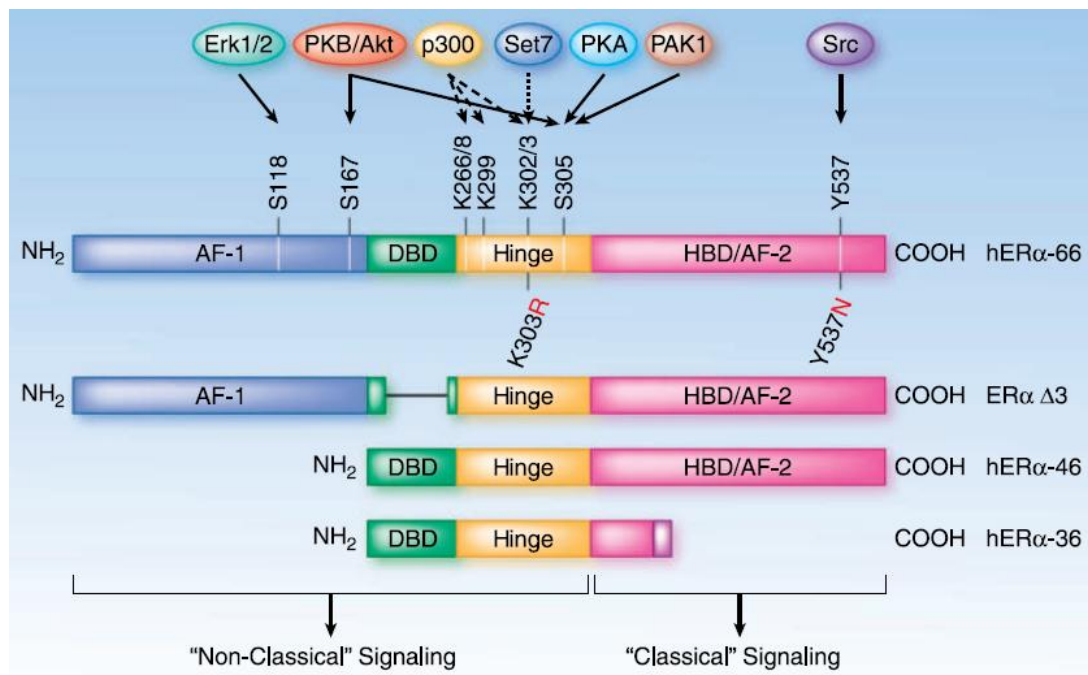
basal transcription machinery to the target gene promoters thereby affecting the transcriptional output [47].

Co-activator binding to the ligand binding domain of the steroid hormone receptors depends on the presentation of the hydrophobic cleft formed by the helices 3, 4, 5 and 12 of ER. The hydrophobic cleft of agonist bound ER is recognized by the LxxLL motif in the co-activator proteins, where L is leucine and x represents any amino-acid. In contrast, no such conformation is adopted by the pure antagonist ICI 182, 780-bound ER, where the long side chain of ICI 182,780 completely disrupts helix 12 interaction with the rest of the LBD. Crystallographic evidence further reveals that the structural basis for trans-hydroxy tamoxifen (OHT) and raloxifene (RAL) antagonism lies in the repositioning of helix 12 over AF2, which then occludes binding of co-activators.

### ***Estrogen Receptor: Post-translational Modifications***

Growth factor signaling leads to numerous downstream phosphorylation events located within the AF-1, DBD, and hinge domains, thus affecting ER $\alpha$  signaling through the “nonclassical” signaling pathways. Post-translational modifications located within the HBD/AF-2 domain affect the “classical” signaling pathway of ER $\alpha$ . In the AF-1 domain, S118 and S167 are phosphorylated by Erk1/2 and Akt, respectively, in the hinge domain, S305 by Akt, PKA, and PAK-1. Phosphorylation of these three sites regulates both ER $\alpha$ 's sensitivity to tamoxifen as well as ligand-independent activation of the receptor [48]. Acetylation at residues K266/8, K299, and K302/3 by p300 also modulates ER $\alpha$  activation. A somatic mutation, K303R, allows ER $\alpha$  to be more highly phosphorylated by PKA and Akt, resulting in estrogen hypersensitivity and endocrine resistance. Methylation, occurring by Set7 interaction with ER $\alpha$ , causes increased receptor stability and a heightened recruitment of ER $\alpha$  to its target genes [49]. In the AF-2 domain, the Y537 site is target of c-Src. The Y537N mutation eliminates this phosphorylation site,

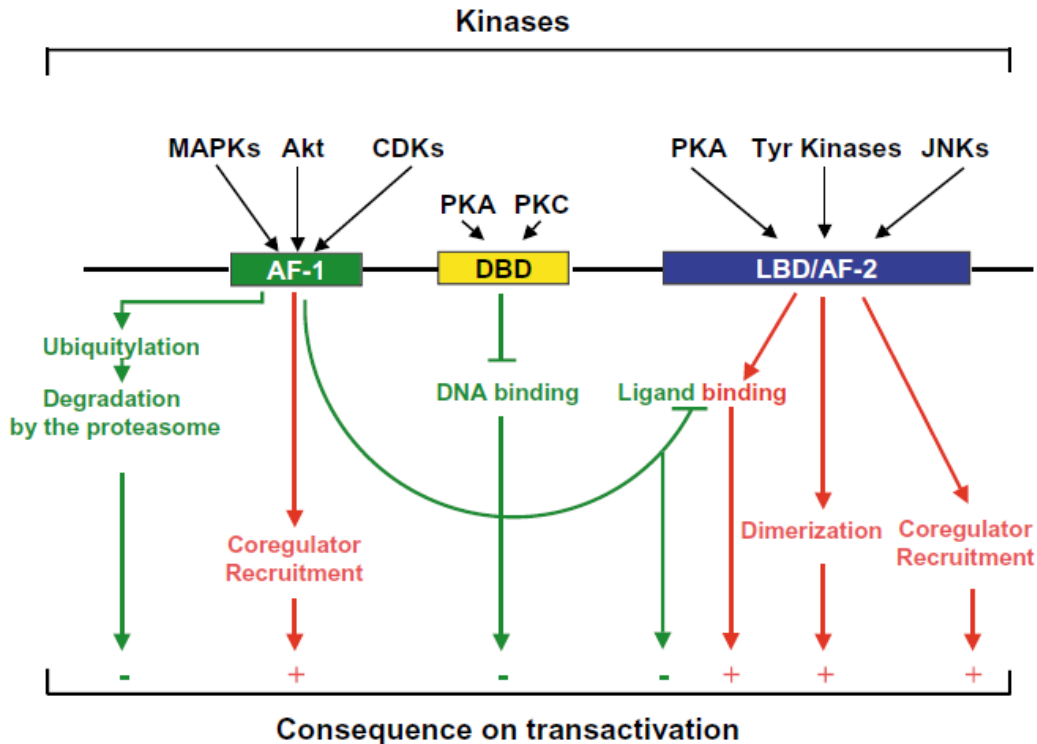
resulting in constitutive receptor activity. ER $\alpha$  can also be modified by alternative splicing at the DNA level, resulting in exon skipping and a truncated protein with a subsequently altered function. For example, in ER $\alpha$   $\Delta 3$ , the third exon is alternatively spliced resulting in a truncated DBD. This isoform does not bind to DNA and is a dominant negative inhibitor to the wild-type ER $\alpha$ . Another truncated ER $\alpha$  isoform is ER $\alpha$ -46, which is missing the AF-1 domain and has been found to localize at the plasma membrane. It acts as a competitive inhibitor to full length ER $\alpha$ . A third alternatively spliced form of ER $\alpha$  is ER $\alpha$ -36, which lacks the AF-1 and AF-2 domains but has an additional 27-amino-acid sequence added at the COOH terminus. This isoform also localizes to the plasma membrane and also acts as a suppressor of full length ER $\alpha$ .



Barone et al., Clin Cancer Res; 16(10) May 15, 2010

**Fig.1.3 Post-translational modifications in Estrogen Receptor**

Schematic representation of Estrogen Receptor  $\alpha$  protein with known post-translational modifications as well as the proteins/enzymes that target the sites for those modifications.



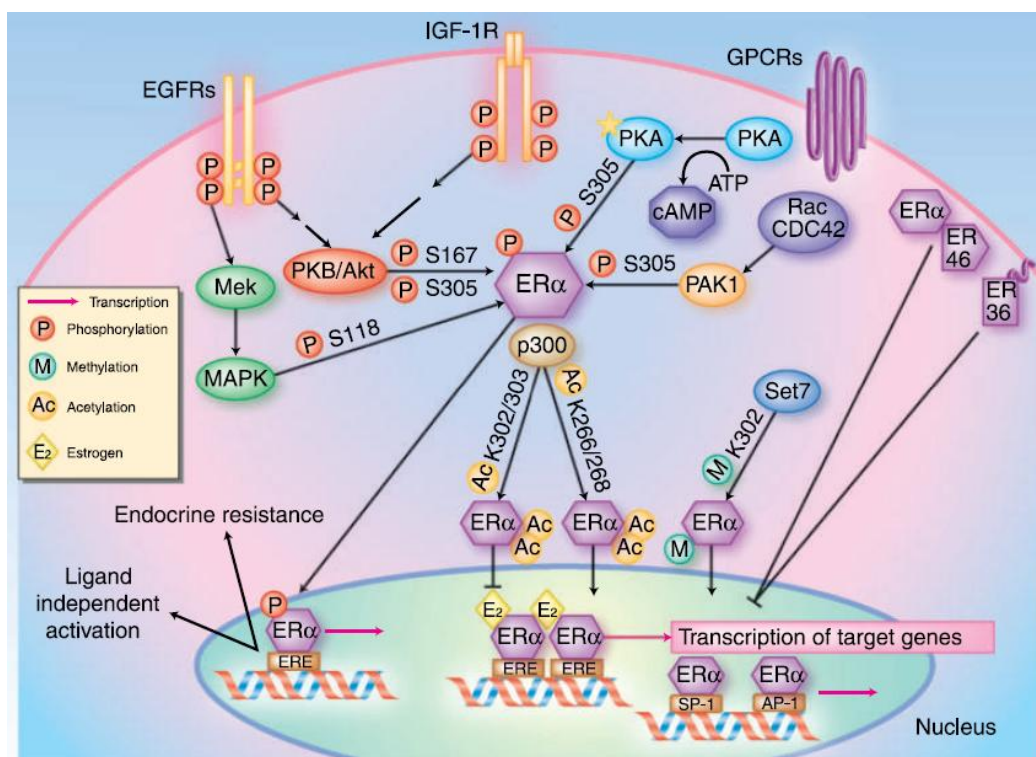
**Fig.1.4 Nuclear Receptor regulation through post-translational modifications**

Schematic representation of the regulation of nuclear receptor function through post-translational modifications by various cellular kinases.

#### ***Estrogen Receptor: Effects of Post-translational Modifications on Levels and Function***

Estrogen receptor mediates transcription of its target genes using two types of mechanisms, known as “classical” and “nonclassical” signaling [50-53]. First, “classical” signaling initiates with the binding of estrogen to estrogen receptor, causing it to bind directly to regions of DNA called EREs, located within transcriptional start sites of estrogen-regulated genes, which subsequently activate transcription of downstream genes. There are several mechanisms of “nonclassical” signaling. The first of these mechanisms is mediated by the signaling of growth factors (such as IGFR and EGFR) and G-protein coupled receptors, through downstream signaling molecules to estrogen receptor [14]. These pathways mediate estrogen receptor’s state of post-transcriptional modification (by affecting its phosphorylation,

acetylation, methylation) and thus its activity, independent of estrogen binding. It is likely that crosstalk of these pathways not only results in estrogen-independent activation of estrogen receptor but also endocrine resistance [54, 55]. Signaling has also been shown to occur through truncated membrane bound forms of estrogen receptor; this signaling is usually inhibitory of full length estrogen receptor activity. Finally, another mechanism of “nonclassical” signaling requires the binding of estrogen receptor to other transcription factors (including SP-1 and AP-1), causing a recruitment of estrogen receptor to transcriptional start sites other than EREs and transcription of downstream genes.



Courtesy: Barone et al., Clin Cancer Res; 16(10) May 15, 2010

**Fig.1.5 Role of Post-translational modifications in Regulating Level and Function of Estrogen Receptor**  
Schematic representation of cross-talk between ERα and growth hormone receptor pathways regulated largely through post-translational modifications of ERα protein by various cellular proteins/enzymes.



### ***Estrogen Receptor: Cellular Turnover and Promoter Dynamics***

Cellular turnover of ER $\alpha$  as well as several other members of the nuclear receptor superfamily, controlled by the ubiquitin proteasome machinery, has since long known to be crucial for their normal functioning and dynamics at the gene promoter. Protein ubiquitination is a dynamic process involving the covalent attachment of ubiquitin (Ub), a small 8.6KDa protein highly conserved in all eukaryotes, to the lysine residues of substrate proteins. The transfer of Ub is a multistep process that involves at least three classes of enzymes: Ub-activating enzymes (E1), Ub-conjugating enzymes (E2) and Ub ligases (E3), of which E3-Ub ligases are the ones that confer substrate specificity by interacting directly with substrate proteins and hence are targets for therapeutic intervention [56, 57], [58].

Studies on nuclear receptors have revealed multiple points of overlap between the proteasome and the receptors. Components of the proteasome coexist with the receptor and their co-regulators in the transcriptional complexes within the nucleus. Receptor interaction with DNA and with co-regulator complexes is dynamic, and release of receptors from chromatin appears to be essential for efficient transcription [59]. We thus investigated the mechanism and role of ubiquitin mediated protein turnover in regulating ER $\alpha$  levels and function and identified Skp2 (S-phase kinase-associated protein-2) as a novel E3-ubiquitin ligase that regulates ubiquitination and turnover of ER $\alpha$ , in breast cancer cells [38, 60]. Skp2, which is an F-box protein (FBP) and substrate recognition component of SCF (Skp1-Cul-1-FBP) ubiquitin ligase complex, is one of the two major ubiquitin ligases that play important roles in cell-cycle regulation, the other being anaphase-promoting complex/cyclosome (APC/C) (Wang et al.). The fundamental importance of SCFSkp2 in regulating cell cycle progression and hence the growth and physiology of breast cancer cells, stems from the fact that it is the key regulator of cell cycle checkpoint proteins and cyclin-dependent kinase (CDK) inhibitors p27 and p21 [61, 62].

### ***Estrogen receptor signaling regulated by ubiquitination***

Estrogens display tissue-selective action that is of great importance in the development of normal breast tissues as well as breast cancer. The interaction of the ligand estrogen with the estrogen receptor, a nuclear hormone receptor, induces transcriptional activity of the receptor. Approximately half of breast cancers are positive for ER $\alpha$  and are thought to be estrogen dependent. ER $\alpha$  provides us not only with a powerful prognostic marker but also a useful target for the treatment of ER-positive breast cancer patients with anti-estrogens. It has been more than three decades since ER was discovered; however, the regulation of its transcriptional activation is still unclear. The ubiquitin–proteasome proteolytic pathway is one mechanism involved in ER signaling. Upon treatment of cells with estradiol, ubiquitination of ER $\alpha$  is enhanced (Nirmala and Thampan, 1995). This ubiquitination is important for degradation of ER $\alpha$  by the proteasome-dependent proteolytic pathway, and it is also required for efficient ER $\alpha$  trans-activation. Treatment of ER $\alpha$  transfected HeLa cells with the proteasome inhibitor, MG132, stabilized ER $\alpha$  levels but impaired ER  $\alpha$ -mediated transcription (Lonard et al., 2000). Mutations in helix 12 of ER  $\alpha$ , the critical core of the AF-2 function of the receptor that interacts with transcriptional coactivators such as SRC-1, abolished ligand-mediated degradation and trans-activation of the receptor (Lonard et al., 2000; Preisler-Mashek et al., 2002). Interestingly, some receptor antagonists including tamoxifen, which is an anti-estrogen used in breast cancer therapy, stimulate proteasome-dependent proteolysis of ER (Preisler-Mashek et al., 2002, Pearce et al., 2003). It is likely that the anti-estrogen effect of such ligand antagonists is, at least in part, mediated by the modification of ubiquitin–proteasome dependent degradation of ER.

A subsequent question that emerges is, what is the E3 ubiquitin ligase in this process? As ligand interaction with ER is the critical step, the ubiquitin ligase may not be very important to the process. However, targeting of a substrate by a ubiquitin ligase is specific, and small molecules inhibiting the

interaction between the ubiquitin ligase and ER  $\alpha$  may be useful as anti-estrogens in breast cancer therapy. Therefore, it is beneficial to determine the E3 ligase responsible, and there are a couple of candidates. First, the HECT ubiquitin ligase, E6-AP, was initially identified as a ubiquitin ligase for p53 in the presence of the E6 protein from human papilloma virus types 16 and 18. It directly interacts with members of the nuclear hormone receptor superfamily [63, 64]. Under normal conditions without E6, E6-AP targets other substrates for ubiquitination, such as Src family members. Furthermore, E6-AP is over-expressed in mouse mammary tumors [65]. The interaction between E6-AP and the nuclear hormone receptor is hormone- dependent and activates transcriptional activity [63, 64]. However, the ubiquitin ligase function of E6-AP is dispensable for its ability to coactivate nuclear hormone receptors [63, 64], suggesting that ubiquitination of ER may not be mediated by E6-AP.

Another candidate could be BRCA1, since BRCA1 mediates transcriptional repression of ER  $\alpha$  [66]. However, this BRCA1 transcriptional repression is ligand-independent [67]. Hence, the bonafide ubiquitin ligase for ER $\alpha$  remains to be determined. Identification of the downstream effectors of ER required for cell proliferation is a critical issue to understand hormone-dependent breast carcinogenesis and to generate treatments for such cancers. One of the genes whose expression is upregulated by ER upon estrogen stimulation is cyclin D1, whose significance has already been discussed. Another important downstream protein is the estrogen-responsive finger protein, Efp. Efp is predominantly expressed in various female organs and is essential for estrogen dependent cell proliferation and organ development [68]. Mice deficient for Efp display underdeveloped uteri and reduced estrogen responsiveness [68]. Interestingly, Efp possesses a RING finger B-box coiled-coil (RBCC) motif and, like other RING finger proteins, displays potent ubiquitin ligase activity [69]. Efp targets the proteolysis of 14-3-3s [69], a p53-inducible gene that inactivates the mitotic CDC2/cyclin B complex by sequestering it in the cytoplasm [70]. Over-expression of Efp in estrogen dependent MCF7 breast cancer cells causes tumor formation in nude mice in the absence of estrogen [69]. Alternatively, estrogen-dependent tumor

growth of MCF7 cells implanted in female nude mice is reduced by treatment with antisense Efp oligonucleotide [69]. These data suggest that Efp is a major downstream target of estrogen. An important interpretation for the breast cancer clinic is that Efp could promote a switch from estrogen dependent to estrogen-independent growth of breast cancer cells. Most estrogen antagonist-sensitive breast cancers switch to being insensitive after several rounds of the hormone therapy. Thus, Efp could be an ideal molecular target for the treatment of such breast cancers.

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## CHAPTER II – SKP2 MEDIATED REGULATION OF CELLULAR LEVEL OF

### ER $\alpha$ IN BREAST CANCER CELLS

#### ABSTRACT

Estrogen receptor alpha (ER $\alpha$ ) is a master regulator of the proliferation and phenotypic properties of breast cancer cells. The regulation of its cellular level is crucial to the effectiveness of endocrine therapies, namely treatment with selective estrogen receptor modulators or with aromatase inhibitors that block signaling by ER $\alpha$ . We found that the E3-ubiquitin ligase, Skp2 (S phase kinase-associated protein-2, that functions in phosphorylation dependent ubiquitination and is a key regulator of check point proteins like p27 and p21), directly interacts with ER $\alpha$  and regulates its ubiquitination and degradation thereby impacting its protein half-life in breast cancer cells. Mutagenesis and deletion studies revealed that the two proteins interact through their N-terminal regions and that the F-box domain of Skp2 is required for ER $\alpha$  degradation.

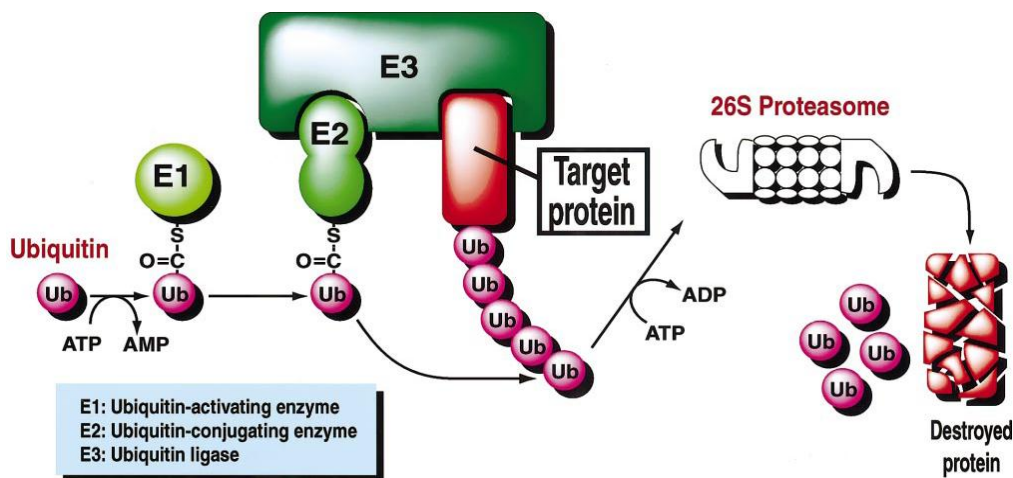
Our examination of many breast cancer cell lines revealed an inverse correlation between the levels of ER $\alpha$  and Skp2 protein. Likewise, in human breast tumors, Skp2 expression was lower in ER-positive than in ER-negative tumors. Consistent with these observations, knockdown of Skp2 in MCF-7 cells resulted in a marked increase in ER $\alpha$  protein, while over-expression of Skp2 caused a rapid loss of ER $\alpha$  protein. Furthermore, characterization of the SCF<sup>Skp2</sup> complex revealed Cul7 and Rbx1 as important components required for the ubiquitin mediated proteasomal degradation of ER $\alpha$  by Skp2. Our findings that highlight a novel SCF<sup>Skp2</sup> complex for the regulation of cellular level of ER $\alpha$  may underlie the inverse relationship between Skp2 and ER $\alpha$  in human breast tumors.

## INTRODUCTION

### *Ubiquitin Proteasome System*

In growing cells, post-translational protein modifications direct the rapid transitions through consecutive cell cycle phases. To cycle unidirectionally, cells can use the destruction of proteins which sustain an existing cell cycle phase, yet prevent onset of the next. Degradation of such cell cycle regulators is typically executed by the 26S proteasome which recognizes the ill-fated proteins by poly-ubiquitin chains. These chains function as proteasome targeting earmarks, formed on one or some of the target's lysine residues. It is thus central to successful cell cycles that the ubiquitination of regulatory proteins is carefully coordinated.

Ubiquitin-labeling requires a three-step process carried out by E1, E2 and E3 enzymes. The E1 “carrier” enzyme charges an E2 enzyme (also called ubiquitin-conjugating enzyme, UBC) with ubiquitin before it can be linked to the substrate. The transfer requires an E3 ubiquitin ligase which captures the substrate and presents it to the ubiquitin-loaded E2 in such a manner that its lysines can be efficiently ubiquitinated [1, 2]. A wide variety of E3-ligases, ranging from monomeric (e.g. Chfr1, a regulator of prophase, or Mdm2, a destabilizer of p53), to large multi-subunit complexes such as the APC/C discussed here influence the cell-cycle. In the control of ubiquitination, either activation of the E3 enzyme or the ability of the E3 to recognize its substrate is rate-limiting. However, just as protein phosphorylation by kinases can be counter-balanced by phosphatases, the extent to which E3 activation results in effective protein ubiquitination is determined by the activity of de-ubiquitinating enzymes (DUBs). In this respect, it is remarkable that ubiquitin chains need a certain length before they can be recognized by the proteasome. This means that an E3 must either stay bound long enough to its substrate to process it multiple times, or needs multiple successive encounters with the substrate before sending it for destruction. Both models leave ample opportunities for de-ubiquitinating enzymes



Courtesy: Kei-Ichi Nakayama et al. Biochem. and Biophys. Res Comm. Vol 282, 4, 2001, 853-860

**Fig.2.1 Ubiquitin-Proteasome Pathway**

Schematic representation of the ubiquitin-proteasome pathway for targeted ubiquitin proteasomal degradation of cellular proteins.

### ***E-3 Ubiquitin Ligases Associated with Cancer***

E3 ubiquitin ligases are classified into several groups. E3 ligases of the HECT-type, single RING-type, SCF-type, ECV-type, and Cul4-base selectively target oncogenic proteins or tumor suppressive proteins and are involved in carcinogenesis or tumor development. The SCF-type E3 ligase controls many cancer-associated proteins including both tumor suppressor proteins and oncogene products. It consists of four components: the invariable subunits Skp1, Cul1, and Rbx1/Roc1 and a variable subunit F-box protein that serves as a receptor for target proteins and thereby determines target specificity. Generally one substrate is regulated by more than one ligase and one ligase targets several substrates, whereas the E3 ligase seems to be substrate selective. Target recognition by the E3 ligase often requires modifications

of the substrates such as phosphorylation. For example, receptor subunits of SCF-type E3 ligases such as Fbw1 ( $\beta$ -TRCP), Fbw7, and Skp2 often recognize their responsible substrate in a phosphorylation dependent manner.  $\beta$ -TRCP recognizes the D-pS-G-(X)<sub>2</sub>+n-pS destruction motif in its target, the serines of which are phosphorylated by specific kinases [3]. The interaction between Fbw7 and its substrates is also dependent on phosphorylation of the substrate within a motif called the Cdc4-phosphodegron (CPD). The eight WD40 repeats in Fbw7 form a  $\beta$ -propeller structure creating a phospho-epitope binding pocket that can recognize phosphorylated CPD. Several reports indicate that the CPD recognized by Fbw7 contains the motif (L)-X-pT/pS-P-(P)-XpS/pT/E/D (where X is any amino acid). Among the many F-box proteins, Skp2 and Fbw7 have been well characterized and have been shown to control the abundance of proteins associated with human cancers. As shown in Figure 2, Skp2 can ubiquitinate various proteins, including p27Kip1, p57Kip2, p130, Tob1, and c-Myc. Skp2 is mainly involved in degradation of tumor suppressor proteins such as Cdk inhibitors and p130 among others. Over-expression of Skp2 is observed in various human cancers. It has been reported that GA-binding protein is involved in transcriptional up-regulation of the Skp2 gene in tumor cells. Moreover, gene amplifications of the Skp2 gene were found in human gastric cancers. Therefore, Skp2 is suggested to be an oncogene. Fbw7 mainly targets the degradation of oncogenic proteins such as Cyclin E, c-Myc, c-Jun, c-Myb, Notch, and mTOR. Moreover, deletion and mutation of Fbw7 is found in human cancers [4]. Conditional inactivation of Fbw7 manifests thymic hyperplasia because of c-Myc accumulation, resulting in the development of thymic lymphoma. Therefore, Fbw7 is believed to be a tumor suppressor gene that contributes to the negative regulation of the cellular content of oncogene products.

type	component	Substrate recognition module	Tumor-associated substrates
HECT		Nedd4-1/2	TβRI Smad2, 4, PTEN
		Smurf1	TβRI Smad1, 2, 5, 7
		WWP1	p63, TβRI, Smad2, 4
		Itch	p63, p73, Smad2
		ARF-BP1 (Huve1)	p53
Single RING		E6AP-E6	p53
		Mdm2	p53, RB
		COP1	p53
		Pirh2	p53, p27
		c-Cbl	EGFR
SCF		BRCA1	RPB8, RNAPII, ER
		Arkadia	Smad7
		TRIM32	Abi2
		Fbw1 (β-TrCP)	β-catenin, IκB, cdc25, Wee1, Period
		Skp2	p27, p57, p130, Tob1, c-Myc
ECV		Fbw7	c-Myc, c-Myb, Cyclin E, c-Jun, Notch, mTOR
		FBX4-αBcrystallin	Cyclin D1
		VHL	HIFα
Cul4			
		Cdt2/PCNA	Cdt1, p21
		CSA	RNAPII
		XPC	Rad23B

Courtesy: Kyoko Kitagawa et al. Cancer Sci | August 2009 | vol. 100 | no. 8 | 1374–1381

### Fig.2.2 Tumor-associated E3 ubiquitin ligases and their substrates

Type variations and their components of E3 ubiquitin ligases associated with tumors are indicated on the left side. They are classified into five types: HECT-type, single RING-type, SCF-type, ECV-type, and Cul4-base. Specific E3 ligases and their reported substrates associated with cancer are also indicated. The green boxes are substrate recognition modules, and yellow boxes indicate the RING active domain that receives ubiquitin from E2.

Breast cancer susceptibility gene (BRCA) 1, a RING finger E3 ligase, plays a crucial role in the DNA damage response. The BRCA1–BRCA1-associated RING domain (BARD) 1 heterodimeric RING finger complex has ubiquitin ligase activity, whereas individually, BRCA1 and BARD1 have very low ubiquitin ligase activities. It has been reported that RNA polymerase II (RNAPII) and RPB8 are polyubiquitinated by BRCA1–BRCA1 in response to DNA damage without a decrease in protein amount. Somatic mutation of BRCA1 or BARD1 is often identified in breast cancer and ovarian cancer. Ubiquitin ligase activity of the

complex is important for the prevention of breast and ovarian cancer development. HECT-type E3 ligases control the TGF $\beta$ -Smad pathway, whereas some HECT-type E3 ligases target degradation of the p53 family of proteins. Because these HECT-type E3 ligases are often over-expressed in human cancers, they may be associated with carcinogenesis or tumor cell growth. E3 ligases with an intrinsic single RING-finger domain such as Mdm2, Pirh2 and COP1 also target p53. These single RING-type E3 ligases are often over-expressed in human cancers and thereby carcinogenesis or tumor cell growth should be promoted. In the next portion of this review, we focus on the degradation mechanism of tumor suppressive proteins and oncogenic proteins.

<i>E3 ligase</i>	<i>Role in breast cancer</i>	<i>Type of the E3 ligase</i>	<i>Target (role in breast cancer)</i>
Skp 2	Oncoprotein, prognostic factor	SCF subunit (F-box protein)	p27 (prognostic factor)
hCdc4	Putative tumor suppressor	SCF subunit (F-box protein)	cyclin E (prognostic factor)
Cul 4A	Overexpressed	Cullin family	DDB2 (damaged DNA repair)
BRCA1	Tumor suppressor	RING finger	Unknown
BARD1	Tumor suppressor	Constitutes RING heterodimer with BRCA1	Unknown
c-Cbl	Unknown	RING finger	ErbB2 (oncoprotein, therapeutic target)
CHIP	Unknown	U-box	ErbB2 (oncoprotein, therapeutic target)
E6-AP	Overexpressed in mouse mammary tumors	HECT type	Unknown in breast carcinogenesis
Efp	Oncoprotein/target of estrogen receptor	RING finger/RBCC motif	14-3-3 $\sigma$ (putative tumor suppressor)
Smurf2	Unknown	HECT type	RNF 11 (overexpressed)

Courtesy: Chen C. et al., Mol Cancer Res 2006; 4 (10) Oct 2006

### ***Fig.2.3 E3 Ubiquitin Ligases with known role in Breast Cancer***

Various E3 ubiquitin ligases with known role in breast cancer along with the proteins that they have been proposed to target.

### ***The Ubiquitin-Proteasome System controls the cell cycle***

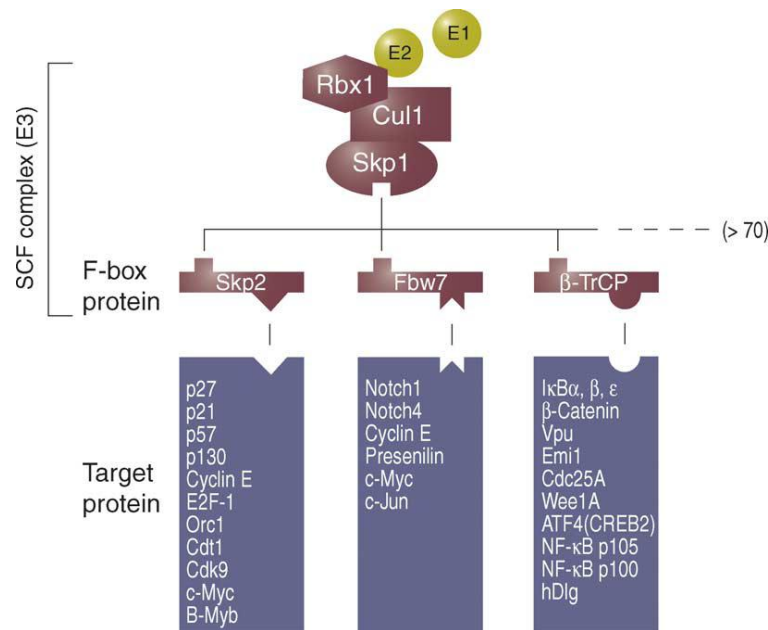
The cell division cycle is regulated primarily by the activity of cyclin-dependent kinases (CDKs) and protein degradation by the ubiquitin–proteasome system (UPS). Each CDK complex contains one of many activating subunits, termed cyclins, the levels of which oscillate during the cell cycle. CKIs (CDK inhibitors), such as p27 and p21, inhibit CDK activity and promote cell cycle arrest and/or delay. SCF



complexes and the APC/C (anaphase-promoting complex/ cyclosome) provide the specific, rapid and timely proteolysis of cell cycle regulators, which ultimately controls CDK1 and CDK2 to finely modulate their activities during cell cycle progression.

Courtesy: David Frescas & Michele Pagano Nature Reviews Cancer 8, 438-449 (June 2008)

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Keiichi I. Nakayama et al. Seminars in Cell & Developmental Biology Vol. 16, 3, 323-333, June 2005

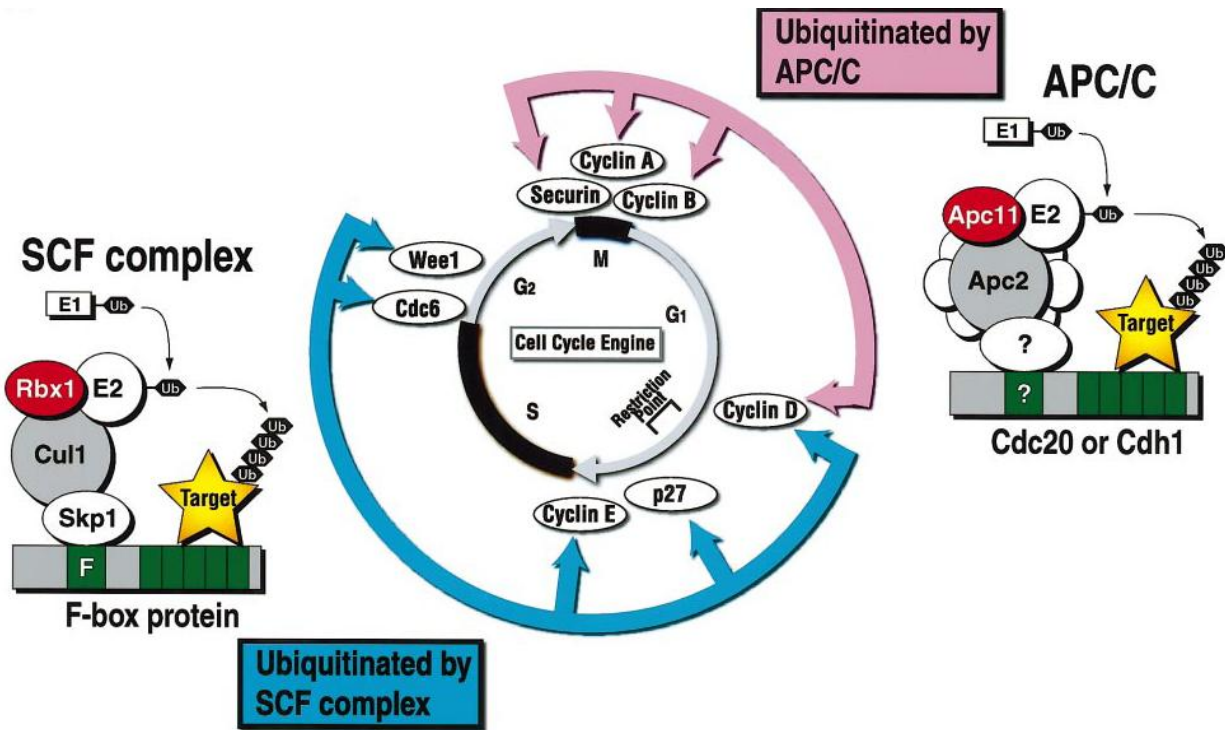
**Fig.2.5 SCF complexes containing Skp2, Fbw7, or β-TrCP target various substrates for ubiquitination**

The SCF complex consists of four components: the invariable subunits Skp1, Cul1 and Rbx1 and a variable F-box protein that serves as a receptor for target proteins and thereby determines target specificity. Among the many F-box proteins, Skp2, Fbw7 and β-TrCP have been shown to control the abundance of cell cycle regulators.

**Major Poly-ubiquitinating Complexes that Regulate Cell Cycle Progression**

Two poly-ubiquitinating E3 complexes that dominantly influence the cell cycle are the SCF (Skp1, Cullin and F-box) and the Anaphase Promoting Complex (APC/C). In contrast to the APC/C, the SCF is considered to be active throughout the cell cycle, recruiting specific targets to an E2 by means of one of several F-box proteins. Importantly, interaction with F-box proteins in many cases depends on the phosphorylation state of the SCF substrate, indicating that substrate-modification and F-box protein availability are the most critical steps in SCF-dependent proteolysis. The APC/C, the largest multi-subunit E3 ligase, is particularly famous for coordinating cell division and sister chromatid separation. The APC/C

first becomes active before that, in prometaphase, and stays active until at least late in G1, so specific recognition of APC/C substrates requires additional levels of control [5, 6], [7, 8].



Courtesy: Kei-Ichi Nakayama et al. Biochem. and Biophys. Res Comm. Vol 282, 4, 2001, 853-860

**Fig.2.6 Cell cycle regulation by the ubiquitin–proteasome pathway**

Roles of two distinct ubiquitin ligases in regulation of the cell cycle. The ordered progression of the cell cycle is regulated by two ubiquitin ligases: the anaphase-promoting complex/cyclosome (APC/C) and the SCF complex. The APC/C is active from late G2 to mid-G1 phase and catalyzes the ubiquitination of mitotic cyclins and securins. In contrast, the SCF complex mediates ubiquitination of G1 cyclins and CKIs.

### **Ubiquitin Proteasome System – Emerging role in Breast Cancer Field**

Breast cancer is the second leading cause of cancer death in American women. It is therefore important to understand the mechanism/s involved in breast cell transformation and to identify novel molecular targets for use in prevention, diagnosis, and therapy. Apart from the accumulation of various genetic

defects, including amplification and concomitant over-expression of certain oncogenes and mutation or loss of various tumor suppressor genes, an overwhelming number of abnormalities have been identified in breast cancer in molecules involved in protein degradation through the ubiquitin proteasome system (UPS). The UPS regulates an enormous range of biological processes, including the cell cycle, apoptosis, transcription, protein trafficking, signaling, DNA replication and repair, and angiogenesis. Consistently, defects in the UPS have already been directly implicated in many diseases, including breast cancer. Well-known examples are the deregulation of the ubiquitin ligases BRCA1, Mdm2, and Skp2. Protein ubiquitination and degradation are usually regulated in a temporal and spatial manner. In most cases, the substrate must be modified first, through mechanisms such as phosphorylation, Prolyl hydroxylation, acetylation, glycosylation, or cleavage before the destruction domain is recognized by E3s. Alternatively, the E3 itself could be activated by posttranslational modification. For example, the anaphasepromoting complex/cyclosome (APC/C) is phosphorylated and activated when cells enter mitosis. Activity of AIP4/ITCH is dramatically activated by c-Jun NH2-terminal kinase-mediated phosphorylation and negatively regulated by Src kinase Fyn-mediated tyrosine phosphorylation. By ubiquitin-mediated proteolysis, cells quickly regulate the cell cycle, transcription, migration, and apoptosis in response to internal and external signaling.

### ***Ubiquitin Proteasome System Regulates Estrogen receptor- $\alpha$ (ER $\alpha$ ) Level***

Estrogen receptor  $\alpha$  (ER $\alpha$ ), a nuclear hormone receptor, is a master transcription regulator of genes that is involved in controlling cell growth and proliferation and hence the main target of endocrine therapies. Approximately 70% of human breast tumors depend on estrogens (the naturally produced cognate ligand for the Estrogen receptor) for proliferation, making them amenable to treatment with drugs such as anti-estrogens (tamoxifen) also known as selective estrogen receptor modulators (SERMs) and/or

aromatase inhibitors (letrozole), which block the signaling by ER $\alpha$ . Targeted therapies involving Tamoxifen or aromatase inhibitors (Osborne, 1999) have seen a steady decline, over the years, in the rate of mortality due to breast cancer. However, these therapies show effectiveness only against ER-positive breast tumors while the ER-negative tumors fail to respond. Even amongst ER-positive tumors, about 40% fail to initially respond to tamoxifen therapy, and of those breast tumors initially responding many eventually develop resistance thereby showing a poor survival and greater recurrence rate. Regulation of cellular level of ER $\alpha$  is therefore, key to the effectiveness of endocrine therapies especially in ER-negative breast tumors.

Cellular turnover of ER $\alpha$  and several other members of the nuclear receptor superfamily is known to be controlled by the ubiquitin proteasome machinery [9, 10], [11-13]. Protein ubiquitination is a dynamic process involving the covalent attachment of Ubiquitin (Ub), a small 8.6KDa protein highly conserved in all eukaryotes, to the lysine residues of substrate proteins. The transfer of Ub is a multistep process that involves at least three classes of enzymes: Ub-activating enzymes (E1), Ubiquitin-conjugating enzymes (E2) and Ubiquitin-ligases (E3), of these E3-ubiquitin ligases being the ones that confer substrate specificity by interacting directly with substrate proteins and hence are targets for therapeutic intervention. We thus investigated the mechanism and role of ubiquitin mediated protein turnover in regulating ER $\alpha$  levels and identified, Skp2 (S-phase kinase-associated protein-2) as a novel E3-ubiquitin ligase that regulates ubiquitination and turnover of ER $\alpha$ , in breast cancer cells. Skp2, which is an F-box protein (FBP) and substrate recognition component of SCF (Skp1-Cul-1-FBP) ubiquitin ligase complex, is one of the two ubiquitin ligases that play key roles in cell-cycle regulation, the other being anaphase-promoting complex/ cyclosome (APC/C) and known to be over-expressed in many cancers including that of the breast. The fundamental importance of SCFSkp2 in regulating cell cycle progression and hence the growth and physiology of breast cancer cells, stems from its regulation of cell cycle checkpoint proteins and cyclin-dependent kinase (CDK) inhibitors p27 and p21 [14].

There have been many studies focused towards understanding how the SCFSkp2 complex works together with the nuclear receptors to execute the cell cycle while maintaining coordination with various cellular processes. Studies on nuclear receptors have revealed multiple points of overlap between the proteasome and the receptors. Components of the proteasome coexist with the receptor and their coregulators, such as kinases and chromatin modifying enzymes, in the transcriptional complexes within the nucleus. Receptor interaction with DNA and with coregulator complexes is dynamic, and release of receptors from chromatin appears to be integral to efficient transcription. We thus investigated the mechanism and role of ubiquitin mediated protein turnover in regulating ER $\alpha$  levels and function and identified, Skp2 (S-phase kinase-associated protein-2) as a novel E3-ubiquitin ligase that regulates ubiquitination and turnover of ER $\alpha$ , in breast cancer cells. SCFSkp2 complex is under tight bimodal regulation by the concerted action of various kinases that modulate its activity by either phosphorylating its components or substrate phosphorylation of its target proteins. There is compelling evidence for the requirement of substrate phosphorylation as a signal for SCFSkp2 mediated protein turnover. We investigated the role of such post-translational modifications in Skp2 mediated ER $\alpha$  turnover. ER is known to be subject to phosphorylation at multiple sites and by multiple kinases like p42/44 MAPK, AKT, protein kinase A, CDKs, the stress related c-Jun NH2-terminal kinase and p38MAPK. We found the stress activated kinase p38MAPK as an important regulator of the Skp2 mediated ER $\alpha$  turnover by phosphorylating ER $\alpha$  at Ser-294 residue [15]. Preclinical studies have shown de novo and/or acquired tamoxifen resistance is associated with increased levels of p38MAPK (Schiff and Osborne 2001). We believe that this resistance could be due to degradation of ER $\alpha$  by Skp2, which is known to be over-expressed in ER-negative tumors, signaled via its phosphorylation by p38MAPK.

The dynamic inverse relationship between ER $\alpha$  and Skp2 expression and involvement of p38MAPK mediated phosphorylation provides a potential to restore ER $\alpha$  expression in ER-negative tumors by either inhibition of ER $\alpha$  interaction with Skp2 or its phosphorylation by p38MAPK using small

molecule or peptide inhibitors [16, 17]. Such inhibitors when used in combination with classical endocrine therapy may hold a greater promise of circumventing endocrine resistance by potentiating ER $\alpha$  levels in ER-positive breast tumors and may be re-expressing it in ER-negative [18-20]. Such combinatorial therapies may therefore offer a valid therapeutic option for the treatment of ER-negative or Her2 over-expressing tumors that do not respond well to endocrine therapy alone.

## **EXPERIMENTAL PROCEDURES**

### ***Antibodies-***

Anti-Skp2 (N-19, H-435, A-2), anti-ER $\alpha$  (HC-20, F-10), anti-Ub (P4D1), anti-HA tag (F-7, Y-11), anti-GST (Z-5) were purchased from Santa Cruz Biotechnology. Anti-Myc tag (2272, 2276), anti-p38MAPK (9212, 9228), anti-phospho (Thr180/Tyr182) p38MAPK (9211, 9216) were from Cell Signaling, anti-flag rabbit and mouse from Sigma. Horseradish peroxidase (HRP)-conjugated donkey anti-goat, donkey anti-mouse and donkey anti-rabbit IgG secondary antibodies were from Santa Cruz Biotechnology.

### ***Cell Culture and Transfections-***

Cos-1 cells were purchased from American Type Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 units per ml of Penicillin and 50  $\mu$ g/ml of Streptomycin at 37°C under 5%CO<sub>2</sub> in humidified air. MCF-7 cells were maintained in growth medium containing Minimum Essential Medium (MEM)-with phenol red supplemented with 5% heat inactivated Calf Serum (CS), 100 units per ml of Penicillin and 50  $\mu$ g/ml of Streptomycin at 37°C in non-CO<sub>2</sub> incubator or treatment medium containing Minimum Essential Medium (MEM)-without phenol red supplemented with 5% Charcoal dextran stripped Calf Serum (CS) at 37°C under 5%CO<sub>2</sub> in humidified air. ZR-75 cells were maintained in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 100 units per ml of Penicillin and 50  $\mu$ g/ml of Streptomycin at 37°C

and 5%CO<sub>2</sub> in humidified air. All transient plasmid transfections were performed using LipofectAMINE™ 2000 Reagent from Invitrogen according to manufacturer's instructions. siSkp2 transfection was performed using Lipitoid reagent kindly provided by Dr.Ronald Zuckermann at the Molecular Foundry, Lawrence Berkeley National Laboratory (23). All other siRNA transfections were performed using Dharmafect transfection reagent from Dharmacon.

### ***Plasmids-***

Skp2 cDNA was purchased by Open Biosystems and cloned into pcDNA3-Flag expression vector. Various Skp2 deletion mutants were generated from this full-length construct by PCR based subcloning. ER $\alpha$  was cloned into either pcDNA3-Flag expression vector or pcDNA3.1-Myc/His expression vector from Invitrogen. Various ER $\alpha$  deletion mutants were generated from the full-length Myc tagged construct by PCR based subcloning. Site directed mutagenesis kit from Stratagene was used to introduce various Ser to Ala or Glu and Lys to Ala or Arg point mutations in pcDNA3-Flag-ER $\alpha$  (full-length). pCMV-ER $\alpha$ / $\beta$  chimeras were generated previously in our laboratory (Sheng et al.). pCMV-HA-Ubiquitin was generated by subcloning from GST-Ubiquitin expression vector (Addgene plasmid 10861) originally generated by Peter Howley. Stub1/CHIP donor plasmid was obtained from plasmID-repository at Harvard (HIP Clone ID# 194919) and later subcloned into pcDNA3.1-Myc/His expression vector before use. pMEV-2HA-p38MAPK-WT (wildtype) and DN (dominant negative) mutant were purchased from Biomyx Technology. Myc-tagged Cul1, Cul2, Cul3, Cul4, Cul5, Cul7 and HA-tagged Rbx1 and Rbx2 were generated by subcloning from cDNA vectors obtained from Addgene. Adenoviral vectors encoding Skp2-WT or  $\Delta$ F-box mutant were constructed by cloning relevant sequences into pAdTrack vector purchased from Stratagene. Details for generation of any of the above mentioned expression vector, is available upon request.



### ***Silencing by Small Interfering RNA (siRNA)-***

MCF-7 or ZR-75 cells seeded onto 6-well plates at a confluence of 40-60% were transfected with siRNA duplexes, siSklp2 (Qiagen) or sip38MAPK (Dharmacon), siCul1 (Dharmacon), siCul2 (Dharmacon), siCul3 (Dharmacon), siCul4A (Dharmacon), siCul4B (Dharmacon), siCul5 (Dharmacon), siCul7 (Dharmacon), siRbx1 (Dharmacon), siRbx2 (Dharmacon) or non-targeting control siGL-3 (Dharmacon or Qiagen). siSklp2 transfection was performed using Lipitoid reagent kindly provided by Dr. Ronald Zuckermann at the Molecular Foundry, Lawrence Berkeley National Laboratory (23, 24). All other siRNA transfections were performed using Dharmafect transfection reagent from Dharmacon.

### ***Adenovirus Construction and Infection for Over-expression of Endogenous Factors-***

cDNAs for the genes of interest, namely Skp2-WT, Skp2 $\Delta$ Fbox, p38MAPK-WT, p38MAPK-DN (dominant negative) and ER $\alpha$  were first cloned into pAdTrack-CMV shuttle vector from Stratagene using PCR based subcloning. DNA from positive clones was linearized by digestion with PmeI and electroporated into electrocompetent BJ5183 cells (Stratagene) already containing plasmidP1 for the recombination event to occur. Recombined DNA was thereafter extracted and transfected into AD293 cells for adenovirus preparation. After rounds of amplification the virus obtained was then purified by CsCl density gradient centrifugation and characterized for its titre and ability to over-express the protein of interest. MCF-7 cells seeded onto 6-well plates at a confluence of 40-60% were thereafter infected with this virus or an equal MOI (Multiplicity of Infection) of a non target control virus followed by ligand treatment (if required). Cells were harvested and processed for RNA and/or protein extraction as per requirement.

### ***Bimolecular Fluorescence Complementation Assay-***

BiFC was performed essentially as described (Hu et al., 2002). Cos-1 cells were transfected with YN and YC fusion constructs alone or in combination and were then incubated for 24h at 37°C in 5% CO<sub>2</sub> and humidified air. Cell nuclei were visualized by CFP-NLS (CFP fused to a nuclear localization signal) which

was co-transfected along with the YN and YC fusion constructs. YFP fluorescence was measured by excitation at 513nm and emission at 527nm. Fluorescence emissions were observed in living cells using an inverted microscope.

#### ***GST Fusion Protein Purification, In Vitro Translation and GST pull down-***

Purification of GST-fused ER $\alpha$  protein from overnight cultures of bacteria and GST-pull down thereafter was performed essentially as described (Gonzalez et al., 2002). In vitro translation of Skp2A and Skp2B and incorporation of S35-labelled methionine (Perkin Elmer) were performed using the TNT T7 Coupled Reticulocyte Lysate System Kit (Promega, Madison, WI). Labelled Skp2A or Skp2B and GST-fused ER $\alpha$  (or GST alone) were incubated together with Glutathione-sepharose beads (Amersham Biosciences) in binding buffer (20mM Tris pH 7.5, 50mM NaCl, 10% glycerol, 10mM NaF, 1% Nonidet P-40, 1mM NaVO<sub>4</sub>, 1X Protease inhibitor cocktail (Roche) and 1mM PMSF) at 4°C for 4h with gentle rotation. The beads were washed three times with the same buffer. Bound proteins were eluted with Laemmli buffer containing SDS supplemented with  $\beta$ -mercapto ethanol and subjected to 10% SDS-PAGE. The gel was then dried and visualized by autoradiography.

#### ***Co-Immunoprecipitation assay-***

Cos-1 cells were seeded onto 6-well plates at a confluence of 80-90% and transfected with plasmids of interest. 24h post transfection cells were harvested, washed with ice-cold PBS and solubilized with 0.5ml CoIP buffer (50mM Tris pH7.5, 150mM NaCl, 0.1% Nonidet P-40, 5mM EDTA, 0.1mM sodium orthovanadate, 1mM dithiothreitol (DTT), 1mM PMSF and 1X Protease inhibitor cocktail from Roche). Immunoprecipitation was carried out using 2-5ug specific antibody by incubation at 4°C overnight with gentle rotation. The precipitated proteins were collected by incubating with Protein-G sepharose (GE Healthcare) for 2h at 4°C with gentle rotation. After low speed centrifugation to remove the supernatant, the agarose was washed with CoIP buffer, boiled in SDS sample buffer and subjected to

SDS-PAGE and Western blotting. In case of MCF-7 cells, endogenous proteins were Immunoprecipitated from lysates obtained from 10cm<sup>2</sup> plates using similar protocol as mentioned above without the involvement of transfections or adenoviral infections for exogenous over-expression of factors.

### ***Ubiquitination assay-***

Cos-1 cells transfected with ER $\alpha$  and other plasmids were pretreated with 10 $\mu$ M MG-132 (Calbiochem, La Jolla, CA) for 5h to block proteasome activity, before harvesting them 24h post transfection. Following harvest, cells were lysed in buffer containing 50mM Tris pH 7.5, 150mM NaCl, 10% glycerol, 5mM EDTA, 1% Nonidet P-40, 0.1mM sodium orthovanadate, 1mM dithiothreitol (DTT), 1mM PMSF and 1X Protease inhibitor cocktail from Roche. Immunoprecipitation was carried using 3 $\mu$ g ER $\alpha$  antibody by incubation at 4°C overnight with gentle rotation. The precipitated proteins were collected by incubating with Protein-G sepharose (GE Healthcare) for 2h at 4°C with gentle rotation. After low speed centrifugation to remove the supernatant, the agarose was washed with CoIP buffer, boiled in SDS sample buffer and subjected to SDS-PAGE and Western blotting with anti-HA antibody (Santa Cruz). In case of MCF-7 cells, endogenous ER $\alpha$  protein was Immunoprecipitated from lysates obtained from 15cm<sup>2</sup> plates following 48h over-expression of either Adeno- $\beta$ -gal or Adeno-Skp2 or Adeno-p38MAPK and 5h treatment with 10 $\mu$ M MG-132 (Calbiochem, La Jolla, CA) to block proteasome activity, using similar protocol as mentioned above and ubiquitinated ER $\alpha$  was detected by anti-Ubiquitin antibody.

## **RESULTS AND EXPERIMENTAL FINDINGS**

### ***Skp2 Regulates ER $\alpha$ Protein Levels and Stability in Mammalian Cells***

ER $\alpha$  and Skp2 protein levels inversely correlate in multiple breast cancer cell-lines (Figure 2.7b) and primary breast tumors from human patients (Figure 2.7a). Next, we hypothesized that Skp2 might be regulating ER $\alpha$  by virtue of its E3-ubiquitin ligase activity. To examine the effect of inhibition of Skp2

expression on ER $\alpha$  turnover, we performed small interfering RNA (siRNA) mediated knockdown of Skp2 in the presence or absence of ligand (E2, TOT and ICI), in MCF-7 and ZR-75 breast cancer cells (Figure 2.9a). The specific siRNA directed against Skp2 reduced Skp2 protein levels to ~10% of that in the scrambled siRNA treated cells and produced a three-fold increase in the steady-state levels of ER $\alpha$  protein. However, this regulation of ER $\alpha$  by Skp2 appeared ligand independent as we observed increased ER $\alpha$  protein in Skp2 knockdown samples irrespective of ligand treatment. We then performed adenovirus mediated over-expression of Skp2-WT (wildtype) or Skp2- $\Delta$ Fbox (dominant negative Skp2 mutant containing the substrate binding LRR but lacking F-box domain) and found a significant decrease in ER $\alpha$  protein only in the presence of over-expressed wildtype Skp2 protein while the dominant negative mutant Skp2 stabilized the receptor. Since the F-box domain is required for interaction of Skp2 with Skp1 in the SCF complex (Weissman, 2001) but not for binding to ER $\alpha$ , this proved the involvement of SCFSkp2 complex in regulating ER $\alpha$  protein level in breast cancer cells. Also this degradation of ER $\alpha$  protein by Skp2 seemed proteasomal dependent as we were able to reverse it in the presence of proteasomal inhibitor MG-132 (Figure 2.9b). We next investigated whether Skp2 regulates the half-life and thus stability of ER $\alpha$  protein in breast cancer cells. We blocked de novo protein synthesis using Cycloheximide (CHX) and then monitored ER $\alpha$  protein levels in the presence of Skp2 adenovirus or control virus. Interestingly, half-life of ER $\alpha$  which was about 4h in the presence of control virus decreased to about 45min with over-expressed Skp2, and by 8h ER $\alpha$  protein was barely detectable (Figure 2.10). Taken together, these results indicate that SCFSkp2 E3-ligase is an important regulator of ER $\alpha$  protein level in breast cancer cells.

### ***Skp2 and ER $\alpha$ Interact in the Nucleus Through their N-termini***

To investigate the mechanism of ER $\alpha$  degradation by Skp2, we examined the association between the two proteins. Co-Immunoprecipitation assay with over-expressed ER $\alpha$  was performed

using both Skp2A and Skp2B (variants of Skp2 protein differing in their C-terminal sequence of the protein) and both Skp2 variants showed interaction with ER $\alpha$  in Cos-1 cells. To further investigate the mechanism of ER $\alpha$  turnover, we examined ligand dependence of the association between the two proteins. Co-Immunoprecipitation assay with ER $\alpha$  over-expressed in Cos-1 cells was performed using both Skp2A and Skp2B (variants of Skp2 protein differing in their C-terminal sequence of the protein), this time in the absence or presence of ligand, Estradiol (E2) or Tamoxifen (TOT). Both Skp2 variants showed interaction with ER $\alpha$  independent of ligand (Figure 2.11a). ER $\alpha$  also co-immunoprecipitated with Skp2 in a ligand independent manner in an endogenous CoIP performed in MCF-7 and ZR-75 breast cancer cells, in the absence or presence of E2 or TOT (Figure 2.11b). We then performed GST pull-down assay to investigate if this interaction between ER $\alpha$  and Skp2 is direct. GST-ER $\alpha$  was expressed and purified from E.coli, immobilized onto glutathione sepharose 4B beads, and incubated with in vitro translated and isotopically (<sup>35</sup>S-Methionine) labeled Skp2A and Skp2B protein. GST was used as a negative control. Protein mixtures bound to glutathione sepharose beads were washed and subjected to SDS-PAGE and analyzed by autoradiography. Only in the presence of GST-ER $\alpha$  were we able to pull-down both Skp2A and Skp2B, but not with GST alone, thereby indicating that ER $\alpha$  and Skp2 interact directly with each other (Figure 2.12a).

We next used bimolecular fluorescence complementation (BiFC) to visualize the intracellular location of the ER $\alpha$ /Skp2 interaction in living cells (Hu et al., 2002). This technique exploits the reconstitution of yellow fluorescent protein (YFP) from non-fluorescent N-terminal (YN) and C-terminal (YC) YFP fragments when they are brought together by two interacting proteins fused to the fragments. On over-expressing ER $\alpha$ -YN and Skp2-YC fusion constructs in Cos-1 cells we found YFP fluorescence localized almost exclusively to the cell nucleus as confirmed by CFP-NLS (cyan fluorescent protein fused to a nuclear localization signal) (Figure 2.12b). No fluorescence was detected in control cells transfected with c-Jun-YN and c-Fos-mut-YC (C-Fos mutant lacking the c-Jun interaction domain), to check for

background fluorescence, thereby demonstrating the specificity of the YFP fluorescence due to ER $\alpha$ -YN and Skp2-YC interaction. To identify the regions of ER $\alpha$  responsible for its interaction with Skp2 we generated a series of Flag-epitope tagged ER $\alpha$  deletion derivatives and expressed them in Cos-1 cells along with wildtype Skp2 (Figure 2.13a and b). Deletion of C-terminal region of ER $\alpha$  did not affect its interaction with Skp2 while truncations in the N-terminal region did, as we found the mutant with N-terminal ABCD region of ER $\alpha$  bound Skp2 just as well as the wildtype receptor. However, we noticed a dramatic loss of interaction with ABC-domain of ER $\alpha$ .

Since, ER $\alpha$  interaction with Skp2 was shown to be nuclear and the nuclear localization signal in ER $\alpha$  is found overlapping C and D-domains of the receptor we wanted to delineate the importance of C-domain with the requirement of a nuclear localization signal. We thus generated an ER $\alpha$  derivative with its AB-domain fused to a nuclear localization signal and found the mutant to interact just as well as the wildtype receptor (data not shown), thereby suggesting that AB domain of ER $\alpha$  to be both essential and sufficient for interaction with Skp2. We then wanted to investigate, what region in Skp2 protein is required for its interaction with ER $\alpha$  we generated a series of Flag-epitope tagged Skp2 deletion derivatives and expressed them in Cos-1 cells along with wildtype ER $\alpha$  (Figure 2.14a and b). Deletion of either the C-terminal regions of Skp2 with or without the Leucine Rich Repeat-box (LRR-box) or the F-box domain had no affect on its interaction with ER $\alpha$ . However, deletion of N-terminal regions of the protein between amino-acids 40-60 appeared to be essential as we observed no interaction between ER $\alpha$  and Skp2 mutant containing all but that sequence. Thus, from our investigations we conclude that ER $\alpha$  and Skp2 directly interact largely in the nucleus and N-terminal regions of both proteins are essential for their mutual interaction.

***Skp2 Mediates Ligand Independent Proteasomal Degradation of ER $\alpha$  with Cul7 and Rbx1 as Part of the SCF<sup>Skp2</sup> complex***

To better understand the cause of loss of ER $\alpha$  protein as a consequence of over-expression of Skp2, we performed ubiquitination assay for ER $\alpha$  in the presence or absence of Skp2. Cos-1 cells were transfected with either ER $\alpha$  alone or ER $\alpha$ /Skp2 together in the presence of over-expressed HA-Ubiquitin and treated with Vehicle (Ethanol), E2 or TOT before they were harvested and processed for co-immunoprecipitation assay with ER $\alpha$  specific antibody. Our results show a significant increase in ubiquitinated ER $\alpha$  in the presence of Skp2 compared to its absence, suggesting involvement of Skp2 in the ubiquitination of ER $\alpha$ . This ubiquitination of ER $\alpha$  by Skp2 also appeared hormone independent as it could be detected both in the absence and presence of E2 or TOT (Figure 2.15a). Also, since Skp2 is a member of the F-box family of E3-ubiquitin ligases that is known to require an N-terminal F-box domain in order to be able to ubiquitinate its targets, we investigated this in its regulation of ER $\alpha$ . We performed ubiquitination assay for ER $\alpha$  in the presence of over-expressed Skp2-WT (wildtype) or Skp2- $\Delta$ Fbox (mutant lacking the F-box domain) and found that the mutant was incapable of ubiquitinating ER $\alpha$  as there was no change in ubiquitinated ER $\alpha$  in the absence of Skp2 or in the presence of over-expressed Skp2- $\Delta$ Fbox mutant, unlike Skp2-WT over-expression (Figure 2.15b).

To establish the occurrence of this mechanism in breast cancer cells we performed ubiquitination assay for ER $\alpha$  in the absence or presence of over-expressed Skp2 through infection of MCF-7 cells with Adenoviral-Skp2 followed by immunoprecipitation of ER $\alpha$  and detection of ubiquitinated form of the receptor using anti-Ubiquitin antibody. We observed a significant increase in ubiquitinated ER $\alpha$  in the presence of over-expressed Skp2 compared to its absence, suggesting involvement of Skp2 in the ubiquitination of ER $\alpha$  in these cells (Figure 2.15c). We next wanted to characterize the SCFSkp2 complex targeting ER $\alpha$  and identify other members of the complex that participate in the ubiquitin-proteasome

mediated degradation of ER $\alpha$  through Skp2. For this we over-expressed Myc-tagged Cullin isoforms (Cul1, Cul2, Cul3, Cul4, Cul5 and Cul7) in Cos-1 cells along with Skp2. Immunoprecipitation with Myc-antibody was performed followed by western blotting for Skp2 to detect which Cullin proteins interact with Skp2. Our data indicates interaction of Skp2 specifically with Cul1, Cul4 and Cul7 (Figure 2.16a). We next over-expressed all the Myc-tagged Cullin proteins in Cos-1 cells, this time with ER $\alpha$  followed by immunoprecipitation with specific ER $\alpha$  antibody and detection of Cullins using Myc-tag antibody. We found ER $\alpha$  to interact specifically with Cul4 and Cul7 proteins (Figure 2.16b). We then performed over-expression of ER $\alpha$  in Cos-1 cells along with HA-tagged derivatives of the two E2 (ubiquitin conjugating enzyme) proteins known to be a part of SCFSkp2 complex, namely Rbx1 and Rbx2. We immunoprecipitated ER $\alpha$  using a specific antibody followed by western blotting for HA-tag to detect which, if any, of the two E2 proteins get co-immunoprecipitated with ER $\alpha$ . We found ER $\alpha$  to interact specifically with Rbx1 and not Rbx2 in these cells (Figure 2.16c).

We next carried out siRNA mediated knockdown of various Cullin and Rbx proteins known to be expressed in MCF-7 cells and monitored the effect of their loss on ER $\alpha$  protein level. Interestingly, loss of Cul4B, Cul7 and Rbx1 resulted in elevated ER $\alpha$  protein (Figure 2.17a). To identify which of these Cullin and Rbx isoforms participate in ubiquitination of ER $\alpha$  by Skp2 we infected MCF-7 cells with either Adeno- $\beta$ -gal (control) or Adeno-Skp2, this time in the presence of either control siGL-3 or specific siRNA targeting Cul1 or Cul2 or Cul3 or Cul4A or Cul4B or Cul5 or Cul7 or Rbx1 or Rbx2. We found that the loss of ER $\alpha$  protein upon over-expression of Skp2 was blocked in the presence of siRNA against Cul7 and Rbx1, thereby implicating the two proteins to play a role in Skp2 mediated degradation of ER $\alpha$  in these cells (Figure 2.17b). Taken together, our data suggests that Skp2 regulation of ER $\alpha$  protein and its stability is due to a hormone-independent turnover of ER $\alpha$  protein by the ubiquitin-proteasome machinery that appears to require Cul7 and Rbx1 to be a part of the SCFSkp2 complex.



## DISCUSSION

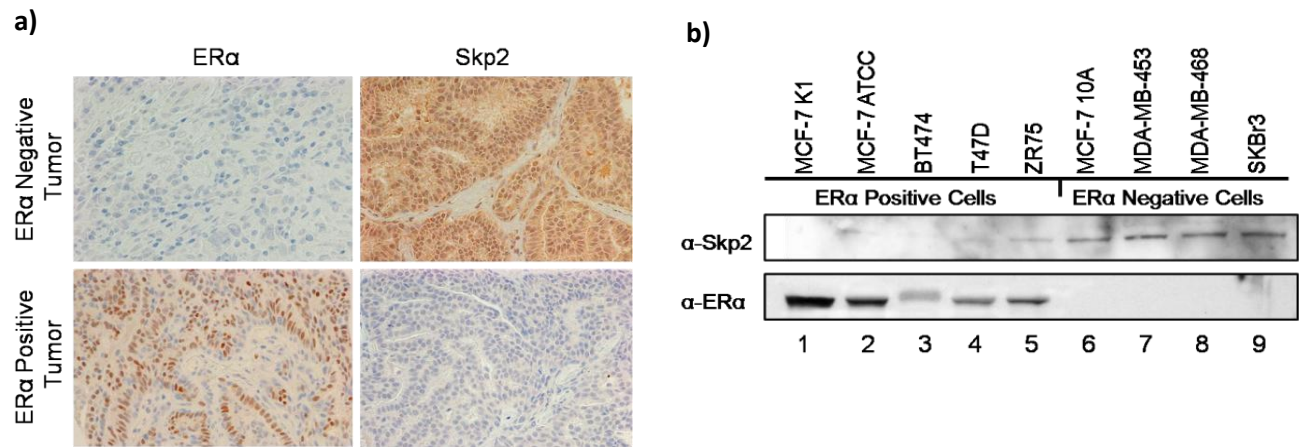
Critical cellular processes are regulated, in part, by maintaining the appropriate intracellular levels of proteins. The ubiquitin-proteasome pathway (UPP) is the most widely known and intensively studied mechanism for the regulation of cellular protein levels (Lee and Goldberg, 1998; Pickart, 2001). In the present study, we have identified Skp2 as a novel E3-ubiquitin ligase that regulates the cellular levels of ER $\alpha$  in breast cancer cells. ER $\alpha$  is a rapidly turned over protein with a half-life of about 4h in MCF-7 cells (Sarff M et al., 1971; Horwitz KB et al., 1978 and 1980; Welsons WV et al., 1993; Kenealy et al., 2000; Alarid ET, 2006). Interestingly, we found over-expression of Skp2 further reduced the half-life of ER $\alpha$  protein in a proteasome dependent manner, thereby supporting the hypothesis that the ubiquitin ligase plays an important role in regulating ER $\alpha$  turnover in these cells. Consistent with these findings we observed a strong inverse correlation between ER $\alpha$  and Skp2 protein levels across multiple breast cancer cell lines and human breast tumor specimen. Regulation of ER $\alpha$  by Skp2 appears to have far reaching functional implications, given the critical roles of the two proteins in regulating cell proliferation and oncogenic transformation (Weiderpass E et al., 2000; Hayashi S.I et al., 2003; Tanaka Y et al., 2003; Yager J.D et al., 2006; Girard G.M et al., 2007; Chae Y.K et al., 2009; Latres E et al., 2001; Signoretti S et al., 2002; Shapira M et al., 2004; Moro L et al., 2006; Sanada T et al., 2004; Traub F et al., 2006; Sonoda H et al., 2006; Gstaiger M et al., 2001; Kamata Y et al., 2005; Lahav-Baratz S et al., 2004; Ma X.M et al., 2006; Yokoi S et al., 2004; Zhu C.O et al., 2004).

In recent years, the involvement of proteasome pathway in the degradation and cellular functions of ER $\alpha$  has been established and many E-3 ubiquitin ligases such as E6AP, BRCA1/BARD1 and CSN5/JAB1 etc. have been implicated in the regulation (Nawaz et al., 1999; Zheng et al., 1999; Urano et al., 2002; Callige M et al., 2005). While a lot of these factors have been shown to be critical regulators of ER $\alpha$  levels and function in breast cancer cells, none of these show specificity towards ER $\alpha$  over a close

homologue ER $\beta$  or several other nuclear hormone receptors like GR, PgR, TR etc., as observed with Skp2 targeted ER $\alpha$  degradation. This makes Skp2 the only reported E-3 ubiquitin ligase specific to ER $\alpha$ , having no effect on ER $\beta$  and therefore an attractive target for pharmacological intervention into ER $\alpha$  actions without affecting ER $\beta$  which is known to often counteract ER $\alpha$  in these cells. Insights into the specificity of interaction of Skp2 with ER $\alpha$  came upon mutational analysis which implicated the N-terminal AB-domain, the region least conserved between the two ERs (Green et al., 1986), in ER $\alpha$  interaction with Skp2. These findings highlight the importance of subtle differences in the sequence of otherwise homologous proteins in directing their regulation in cells through unique mechanisms.

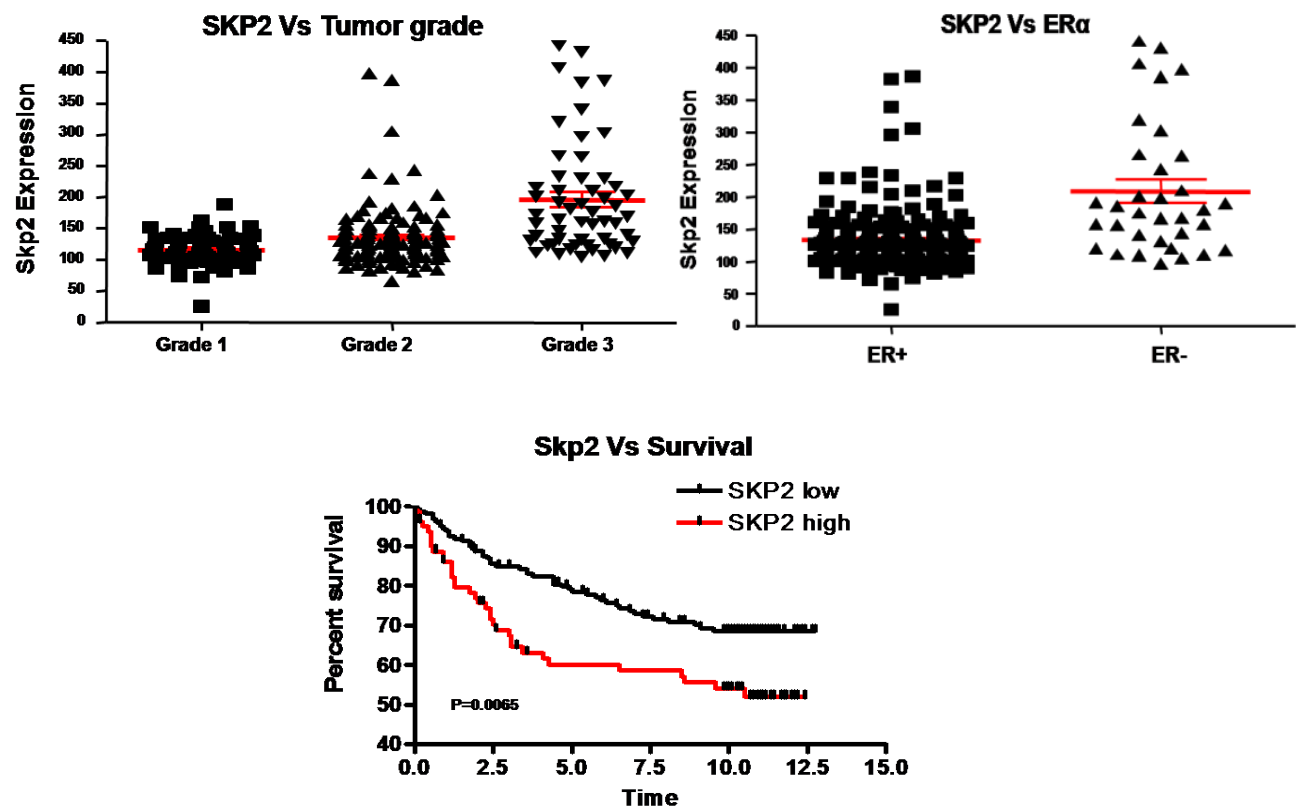
To gain insights into the underlying mechanism for the turnover of ER $\alpha$  by Skp2, we attempted to identify other components of the SCFSkp2 machinery that might be critical for this regulation. In a siRNA screen for various Cullin and Roc protein isoforms we identified Cul-4B, Cul-7 and Rbx-1 as critical regulators of ER $\alpha$  levels in breast cancer cells. Although all three of these factors were found in complex with both Skp2 and ER $\alpha$ , only Cul-7 and Roc-1 were indispensable for Skp2 mediated ER $\alpha$  turnover, while Cul-4B regulation of ER $\alpha$  levels appeared to be independent of Skp2. Surprisingly, Cul-1 which has been suggested to be the dominant partner of Skp2 in the SCFSkp2 complex had no effect on ER $\alpha$  levels in these cells suggesting the regulation of ER $\alpha$  to be through alternate SCFSkp2 complexes containing Cul-7 instead of Cul-1 as the cullin adaptor protein, which according to recent reports has been documented for the regulation of other Skp2 targets in breast cancer cells (Skaar J.R et al., 2007). Our findings that implicate this unique Cul-7 containing SCFSkp2 complex in highly specific and efficient ubiquitination of ER $\alpha$  by Skp2, suggest it to be an attractive target for drug designing for regulating cellular levels of ER $\alpha$  in breast cancer cells.

## FIGURES



**Fig.2.7 Skp2 and ER $\alpha$  Protein Levels Inversely Correlate in Human Breast Tumors and Breast Cancer Cells**

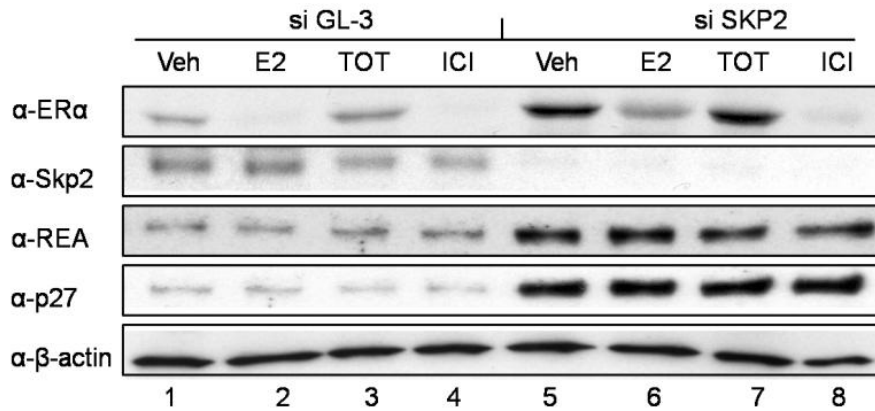
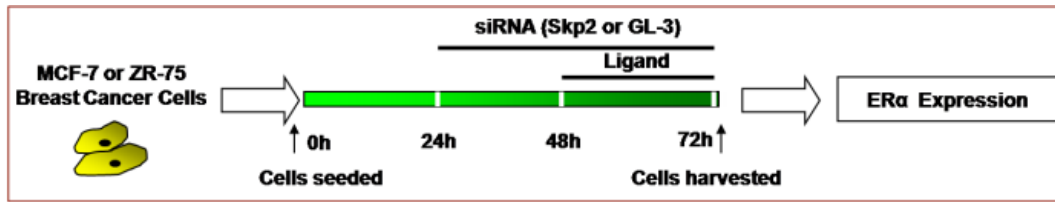
a) Immuno-histochemical staining for Skp2 and ER $\alpha$  protein was performed on paraffin embedded sections of human breast tumor obtained from breast cancer patients using Diaminobenzidine (DAB) to stain the gene of interest and hematoxylin to counterstain. Shown are 20X magnification images obtained from this analysis. b) Relative expression levels of Skp2 and ER $\alpha$  proteins were analyzed in the indicated breast cancer cell-lines by performing SDS-PAGE followed by western blot analysis.



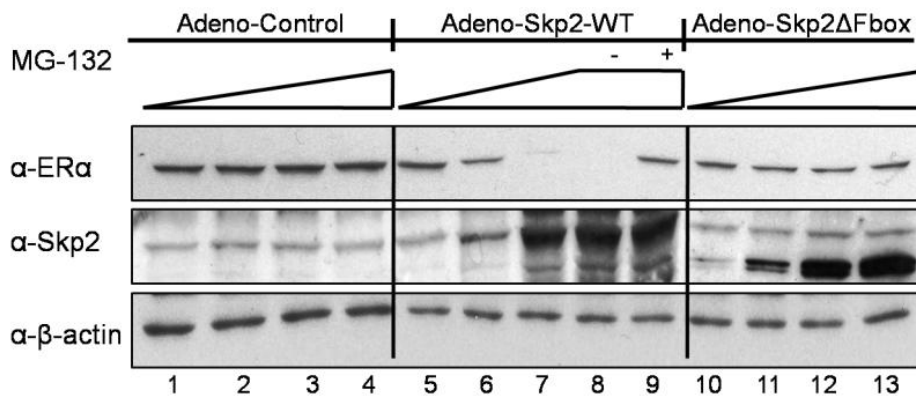
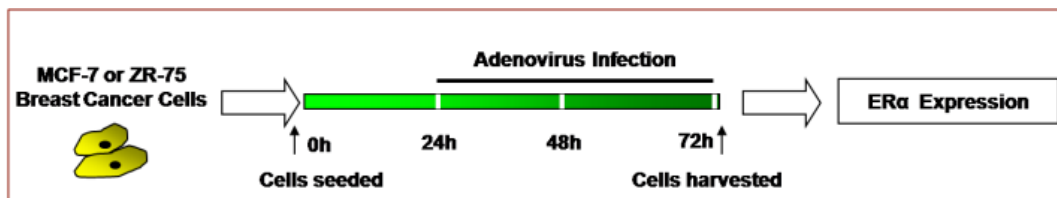
**Fig.2.8 Skp2 Level Shows Strong Correlation with ER $\alpha$  Level, Tumor Grade and Patient Survival in Human Breast Tumor Specimens**

Microarray data analysis: ER $\alpha$ -positive and ER $\alpha$ -negative human breast tumors (Wang et al dataset).

a)



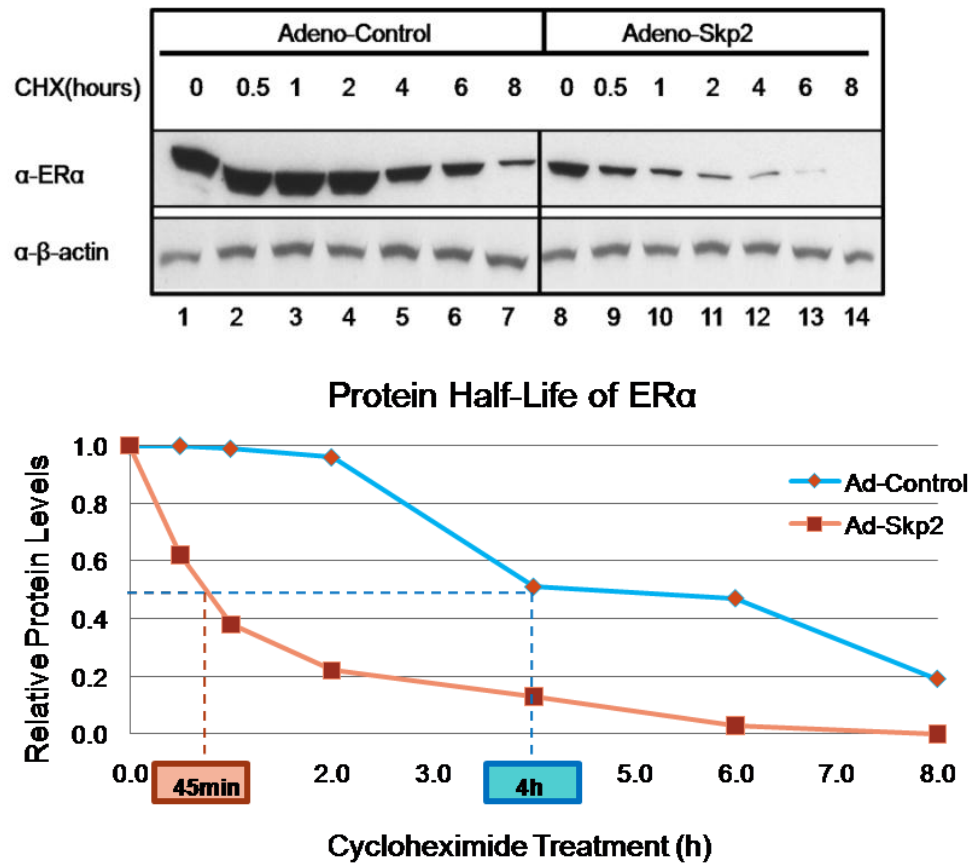
b)



**Fig.2.9 Skp2 Regulates ERα Protein Levels and Stability In Mammalian Cells**

a) MCF-7 cells were transfected with either control siRNA (100nM) or specific siRNA targeting Skp2 (100nM) and 24h post transfection, cells were treated with either Veh (0.1% EtOH), E2 (10nM), TOT (1μM) or ICI (1μM) and lysed after 24h of treatment. b) Effect of over-expression of Skp2 on ERα protein was studied by infecting MCF-7 cells with Adeno-Control, Adeno-Skp2 or Adeno-Skp2-ΔFbox expressing virus followed 72h post infection by treatment with 20μM MG-132 for 5h. In both a) and b) protein was

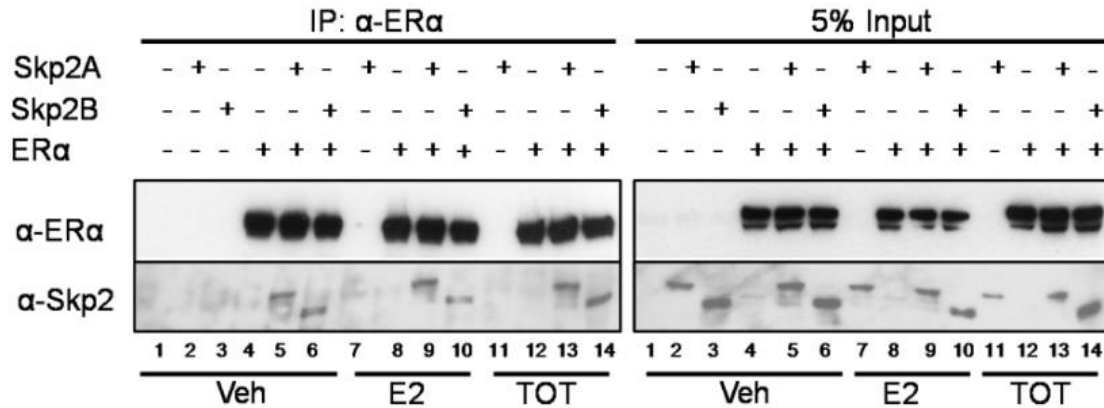
harvested using RIPA buffer and 20ug of total cell lysate was subjected to SDS-PAGE followed by western blot analysis.



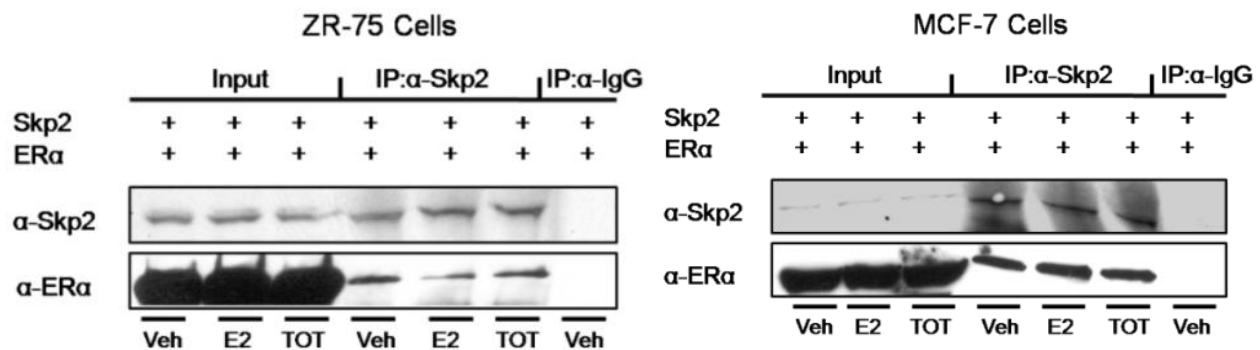
**Fig.2.10 Skp2 Regulates ERα Protein Levels and Stability In Mammalian Cells**

Effect of Skp2 on ERα protein stability was studied by treating MCF-7 cells with Cycloheximide (50ug/ml) for various indicated times to block the de novo protein synthesis, 48h post infection with either Adeno-Control or Adeno-Skp2. Cells were thereafter harvested for protein extraction followed by SDS-PAGE and western blot analysis.

a)

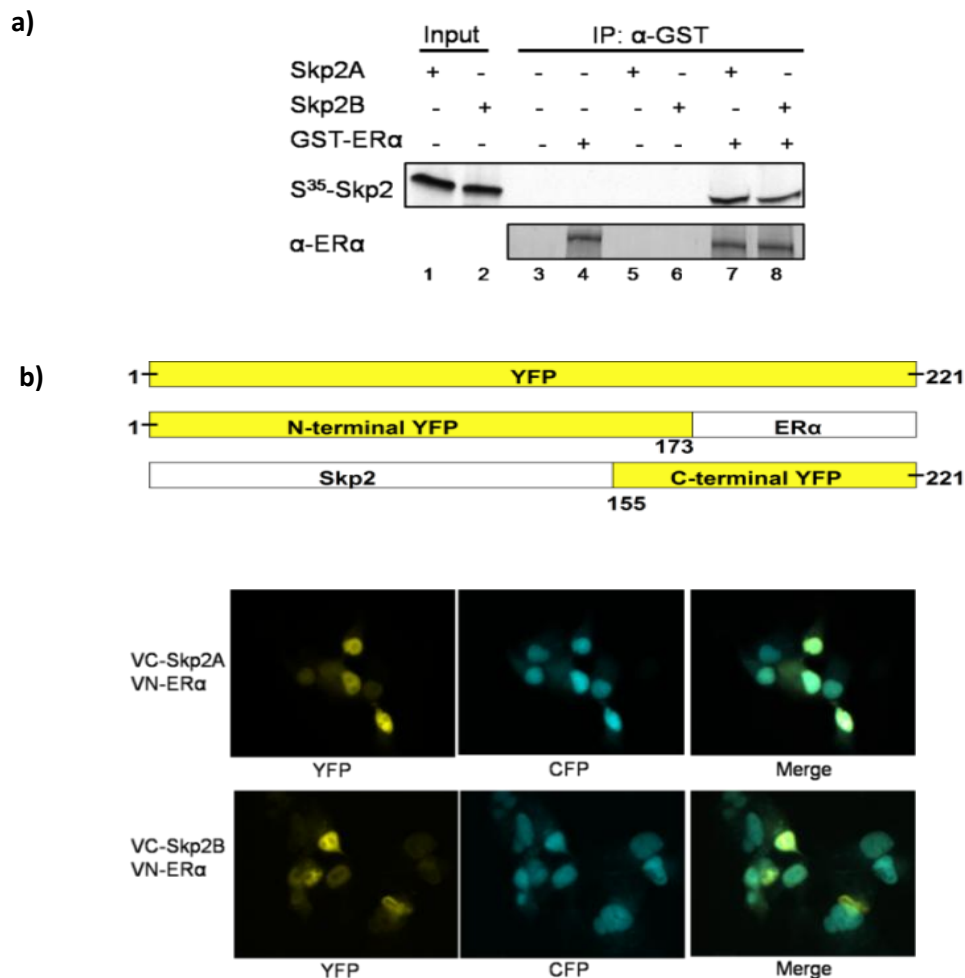


b)



**Fig.2.11 Skp2 and ER $\alpha$  Interact in a Ligand Independent Manner**

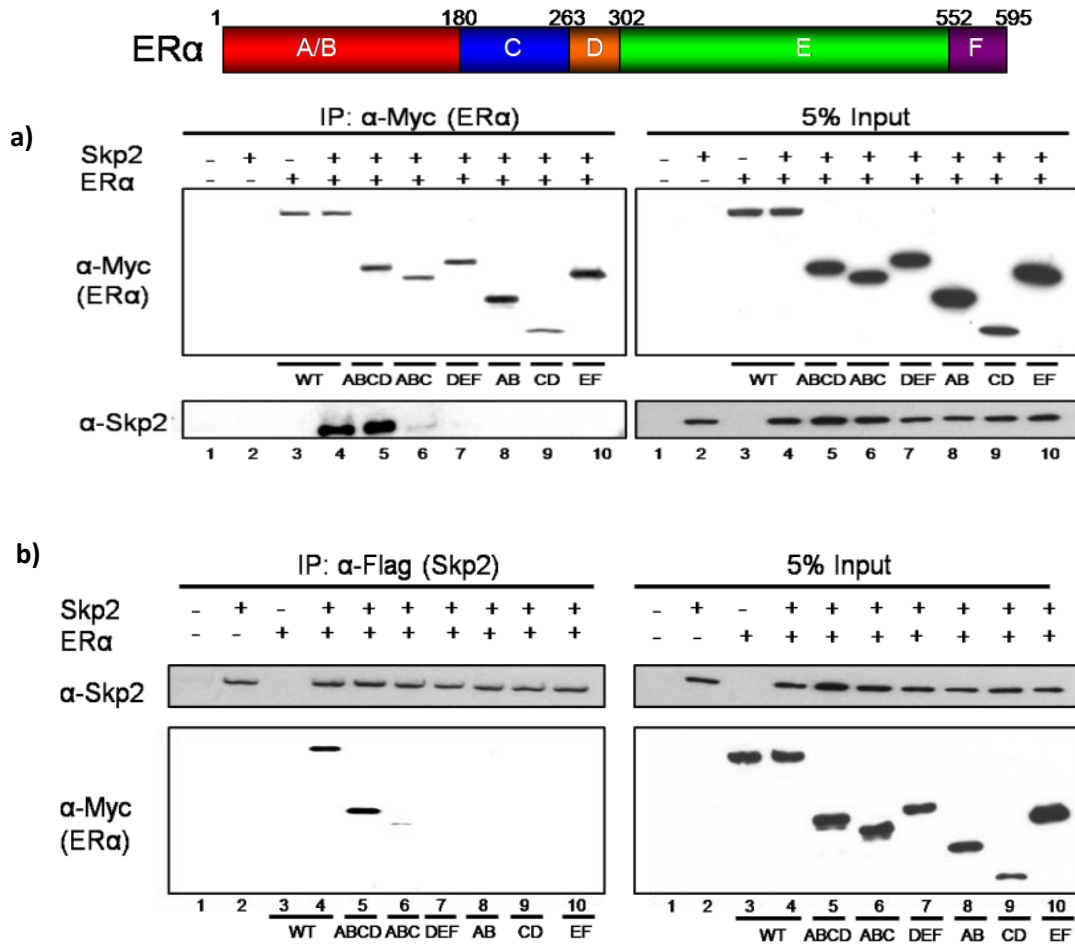
a) Co-immunoprecipitation (CoIP) of exogenous ER $\alpha$  and Skp2. Cos-1 cells were transfected with Skp2A or Skp2B expression plasmid along with that for ER $\alpha$ . Cells were treated with Veh (0.1% EtOH), E2 (10nM) or TOT (1 $\mu$ M) for 4h before they were lysed and harvested 24h post transfection and cell lysates subjected to immunoprecipitation using anti-ER $\alpha$  antibody followed by SDS-PAGE and western immunoblotting with anti-Skp2 or anti-ER $\alpha$ . b) Co-immunoprecipitation (CoIP) of endogenous ER $\alpha$  and Skp2. MCF-7 or ZR-75 cells were weaned for 4 days in treatment media supplemented with charcoal dextran stripped calf serum and thereafter seeded in 10cm plates at 80-90% confluence followed by treatment with either Veh (0.1% EtOH), E2 (10nM) or TOT (1 $\mu$ M) for 1h. Cell lysates were subjected to immunoprecipitation with antibody against Skp2 or a non-specific IgG control, followed by SDS-PAGE and immunoblotting with anti-Skp2 or anti- ER $\alpha$ .



**Fig.2.12 Skp2 and ER $\alpha$  Directly Interact in the Nucleus**

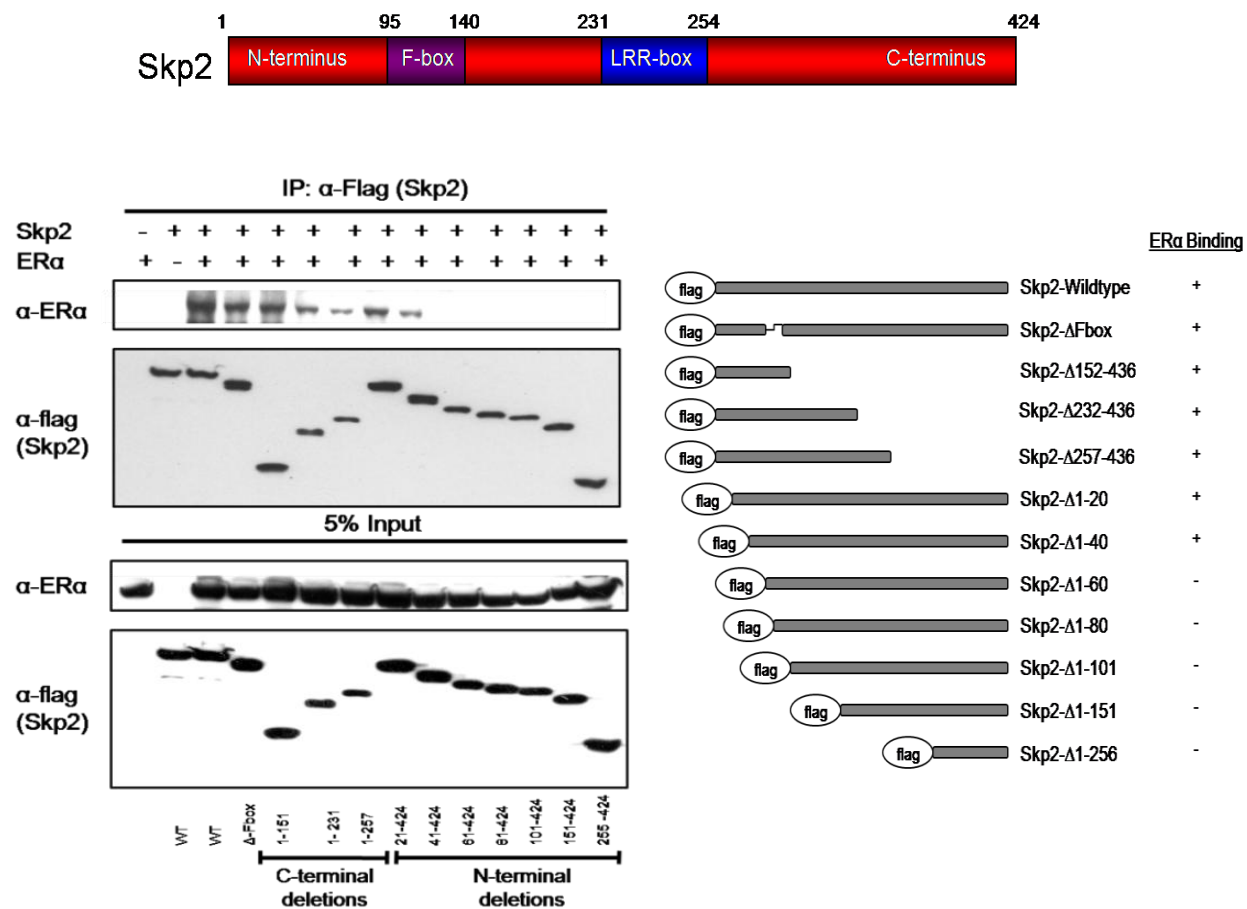
a) In vitro GST pull-down assay was performed using GST fused ER $\alpha$  and in vitro translated S35-Skp2 protein. The input of GST fusion protein was analyzed by SDS-PAGE followed by coomassie blue staining and/or Western blotting. Immunoprecipitated-Skp2 was analyzed by SDS-PAGE followed by autoradiography due to the radioactive label while immunoprecipitated GST fusion protein was monitored simply by SDS-PAGE followed by western blotting. b) Bimolecular fluorescence complementation (BiFC) analysis of ER $\alpha$ -Skp2 interaction in living cells. Cos-1 cells were transfected with YN-ER $\alpha$  (ER $\alpha$  fused to N-terminal part of YFP) along with either YC-Skp2A or YC-Skp2B (Skp2A or Skp2B fused to C-terminal part of YFP) fusion constructs, as indicated, and the cells were analyzed to monitor YFP fluorescence using confocal microscopy. CFP fused with nuclear localization signal was used to visualize the cell nuclei.





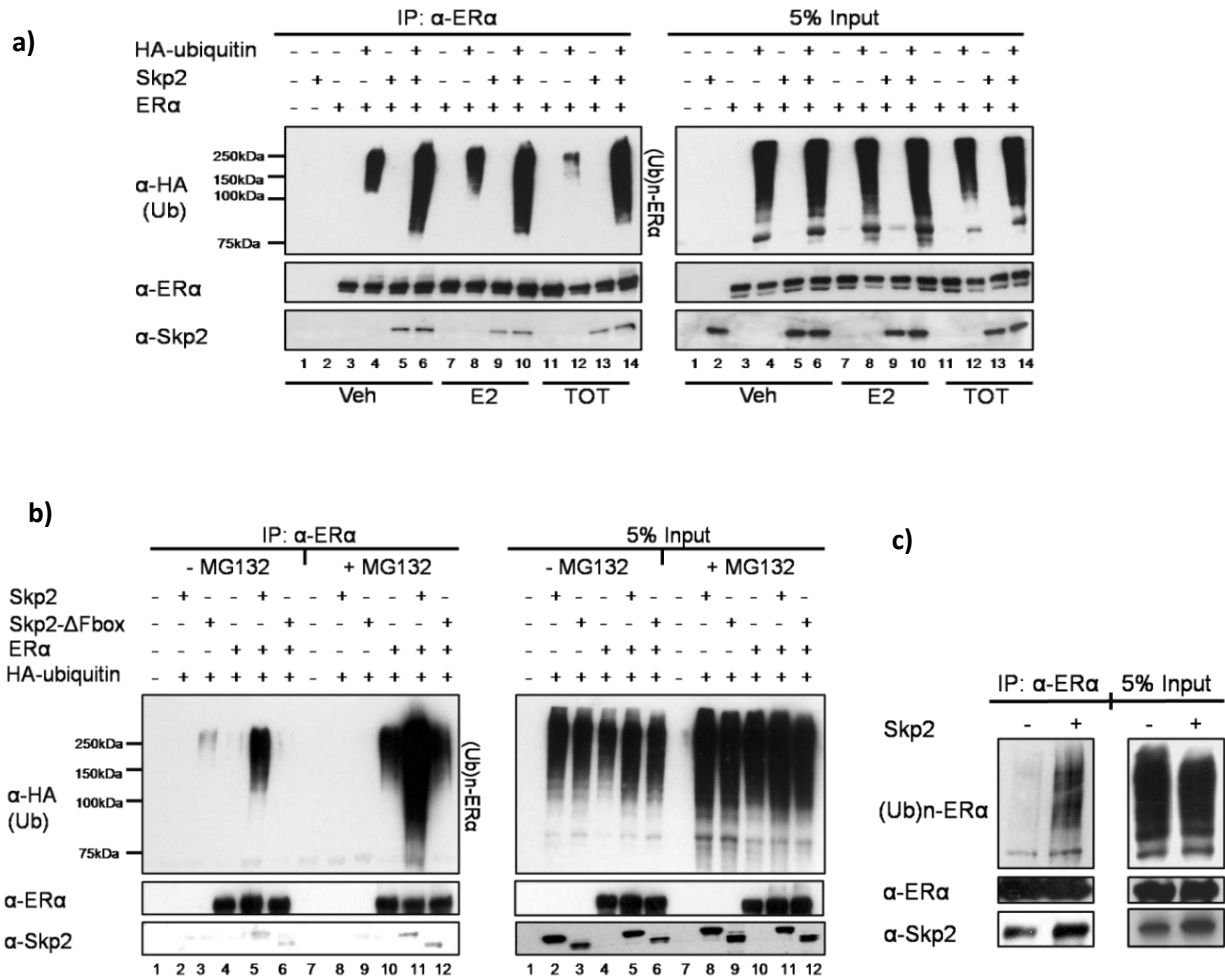
**Fig.2.13 ERα Interacts with Skp2 Through Its N-terminus**

a) Cos-1 cells were transfected with expression plasmids for Flag-Skp2 along with Myc tagged wildtype-ERα or various deletion mutants of ERα (also Myc tagged), as indicated. Cells were lysed 24h post transfection and subjected to immunoprecipitation using anti-Myc antibody to pull down ERα and its truncated forms respectively, followed by SDS-PAGE and immunoblotting with anti-Skp2 and anti-Myc antibodies. b) CoIP assay similar to (a) except this time cell lysates were subjected to immunoprecipitation using anti-Flag antibody to pull down Skp2 followed by SDS-PAGE and immunoblotting with anti-Skp2 and anti-Myc (ERα ) antibodies.



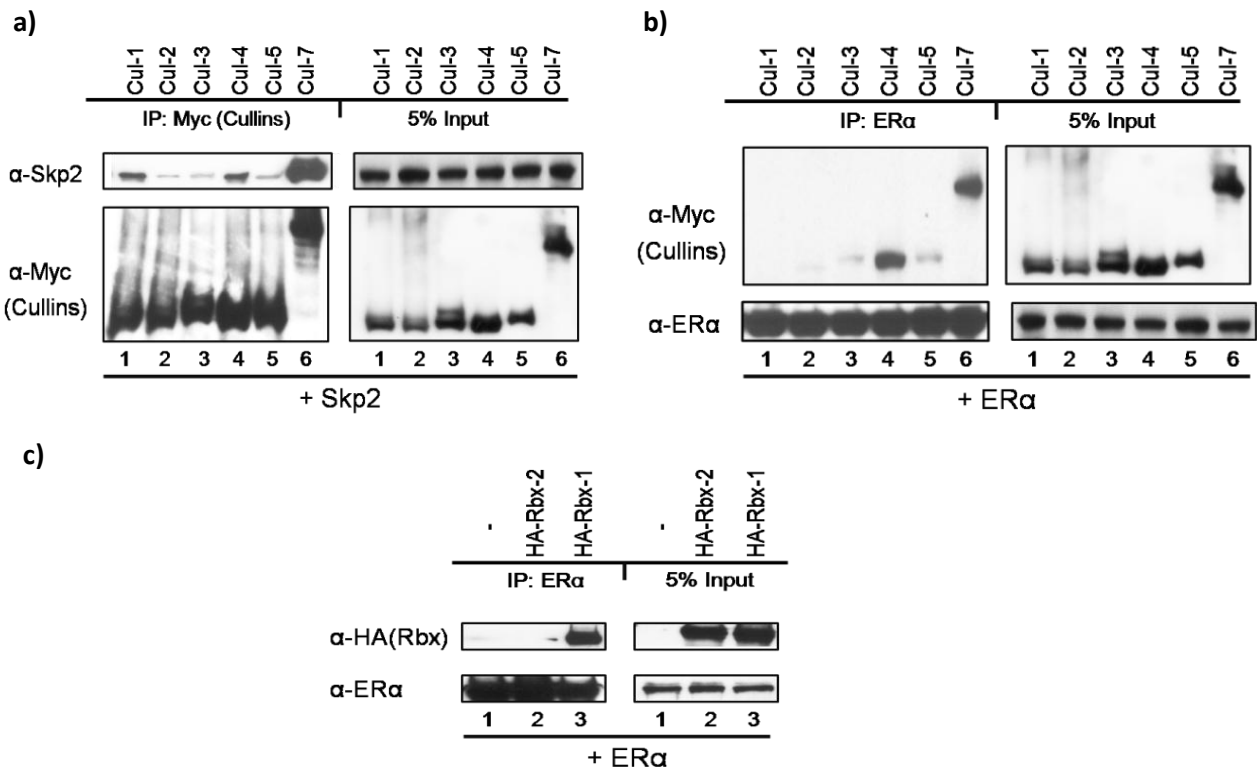
**Fig.2.14 Skp2 Interacts with ERα Through Its N-terminus**

Cos-1 cells were transfected with ERα expression plasmid with no tag along with Flag tagged wildtype-Skp2 or various N or C-terminal deletion mutants, as indicated. Cells were lysed 24h post transfection and subjected to immunoprecipitation using anti-Flag antibody to pull down Skp2 and its truncated forms respectively, followed by SDS-PAGE and immunoblotting with anti-Flag and anti-ERα antibodies.



**Fig.2.15 Skp2 Mediates Ligand Independent Proteasomal Degradation of ER $\alpha$**

a) Cos-1 cells transfected with Skp2, ER $\alpha$  and HA-Ubiquitin (HA-Ub) were pretreated with MG-132 (10 $\mu$ M) for 1h followed by treatment with Veh (0.1% EtOH), E2 (10nM) or TOT (1 $\mu$ M) for 4h before lysis, 24h post transfection. Cell lysates were immunoprecipitated using anti- ER $\alpha$  followed by SDS-PAGE and western blotting for ER $\alpha$ , Skp2 and HA. b) Cos-1 cells transfected with Skp2 or  $\Delta$ -F-box-Skp2 along with ER $\alpha$  and HA-Ubiquitin (HA-Ub) were treated with MG-132 (10 $\mu$ M) for 5h before lysis 24h post transfection followed by immunoprecipitation using anti-ER $\alpha$  antibody, SDS-PAGE separation and immunoblotting for ER $\alpha$ , Skp2 and HA. c) Endogenous Ubiquitination assay. MCF-7 cells were subjected to adenovirus mediated over-expression of either Adeno- $\beta$ -Gal (Control) or Adeno-Skp2 followed 24h after by treatment with proteasome inhibitor MG-132 (10 $\mu$ M) for 5h, subsequent harvest and immunoprecipitation using specific ER $\alpha$  antibody followed by SDS-PAGE separation and western blotting with anti-Ubiquitin (Santa Cruz) to detect ubiquitinated ER $\alpha$ , anti-ER $\alpha$  and anti-Skp2.

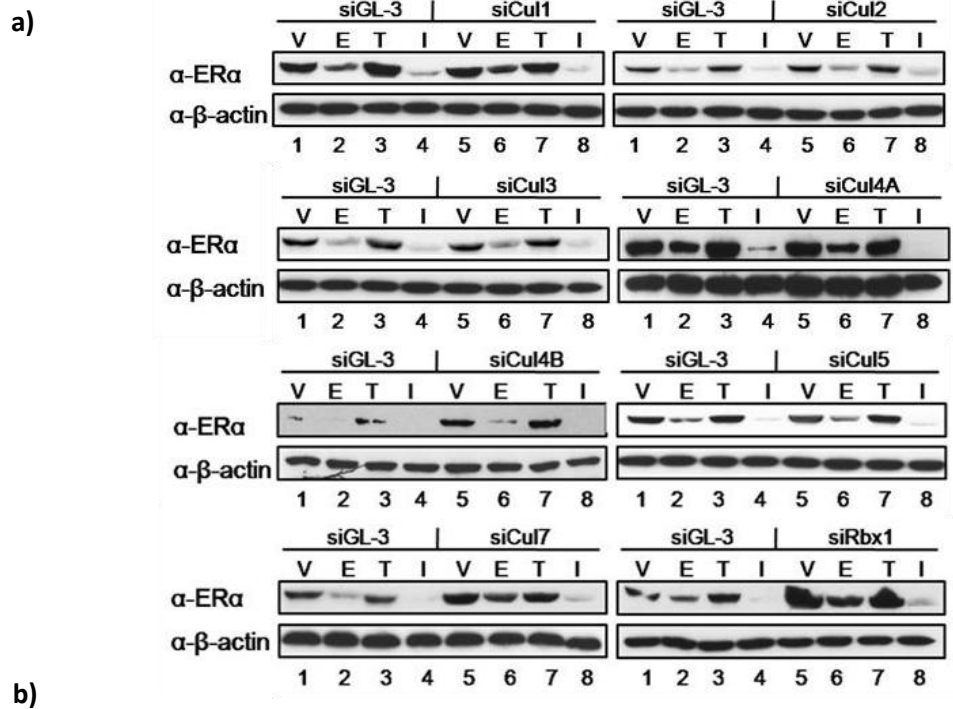


**Fig.2.16 Skp2 and ERα are in Complex with Cul7 and Rbx1**

a) Cos-1 cells transfected with Flag-Skp2 along with Myc-tagged Cullin protein isoforms were lysed 24h post transfection and subjected to immunoprecipitation using anti-Myc antibody to pull down Cullin proteins, followed by SDS-PAGE separation and immunoblotting with anti-Skp2 and anti-Myc antibodies.

b) Cos-1 cells transfected with Flag-ERα expression plasmid along with Myc-tagged Cullin protein isoforms were lysed 24h post transfection and subjected to immunoprecipitation using anti-ERα antibody to pull down ERα and associated proteins, followed by SDS-PAGE separation and immunoblotting with anti-Myc and anti-ERα antibodies.

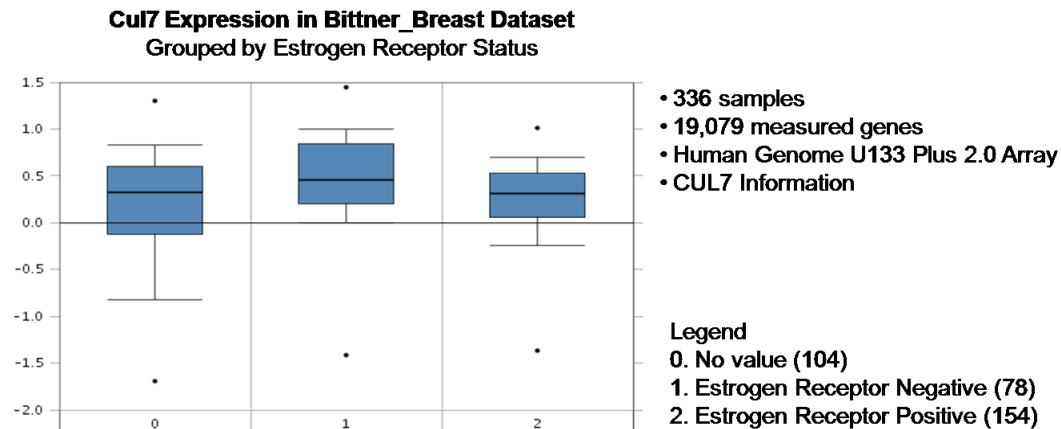
c) Cos-1 cells transfected with Flag-ERα expression plasmid along with HA-tagged Rbx1 or Rbx2 were lysed 24h post transfection and subjected to immunoprecipitation using anti-ERα antibody to pull down ERα and associated proteins, followed by SDS-PAGE separation and immunoblotting with anti-HA and anti-ERα antibodies.



**Fig.2.17 Skp2 Mediates Ligand Independent Proteasomal Degradation of ERα Through Cul7 and Rbx1**

a) MCF-7 cells transfected with either siGL-3 (control siRNA) or siRNA directed against various Cullin protein isoforms (as indicated) or Rbx1 were treated with Veh (0.1% EtOH), E2 (10nM), TOT (1μM) or ICI (1μM) for 24h and thereafter subjected to lysis and protein extraction using RIPA buffer followed by SDS-PAGE and western blotting for ERα or β-actin (loading control). b) MCF-7 cells transfected with either siGL-3 (non-targeted control siRNA) or siRNA directed against various Cullin isoforms (as indicated) or Rbx1 were infected with Adenovirus for either β-Gal (control) or Skp2 and harvested after 48h from the time of adenovirus infection. Cell lysates were subjected to lysis and protein extraction using RIPA buffer followed by SDS-PAGE and western blotting for ERα or β-actin (loading control).

## SUPPLEMENTARY FIGURES



### S1. Oncomine Plot: Cul-7 mRNA is Over-expressed in ER $\alpha$ Negative Human Breast Tumors.

Box-plot from Bittner\_Breast dataset of 336 human breast tumor samples, part of Oncomine database, grouped according to ER $\alpha$  status, showing higher expression of Cul-7 gene in ER $\alpha$  negative tumors (78 samples) compared to ER $\alpha$  positive (154 samples), by microarray analysis (Human Genome U133 Plus 2.0 array).

## ACKNOWLEDGEMENTS

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# **CHAPTER III – REGULATION OF SKP2 DRIVEN ER $\alpha$ TURNOVER THROUGH p38MAPK SPECIFIC PHOSPHO-DEGRON CODE ON ER $\alpha$**

## **ABSTRACT**

Estrogen receptor alpha (ER $\alpha$ ) is a master regulator of the proliferation of breast cancer cells and a key target of endocrine therapy. ER $\alpha$  however is a protein with much shorter half-life when compared to other nuclear hormone receptors. Understanding the regulation of its cellular level therefore is crucial to the effectiveness of endocrine therapies. We found that the E3-ubiquitin ligase, Skp2 directly interacts with ER $\alpha$  and regulates its ubiquitination and degradation in breast cancer cells. Since Skp2 is known to select its targets based on their specific phospho-code, we investigated this in the targeting of ER $\alpha$  for degradation. Of the multiple ER $\alpha$  phosphorylation sites, our data demonstrate that phosphorylation of Serine-294 is essential for ER $\alpha$  ubiquitination by Skp2. Bioinformatic analysis of the Ser-294 containing region suggested it to be a putative Cdk2 or p38MAPK target and in-vitro kinase assay provided evidence for phosphorylation of ER $\alpha$  at Ser-294 by p38MAPK. Interestingly, only in the presence of specific p38MAPK inhibitor or dominant negative p38MAPK were we able to block Skp2 mediated ER $\alpha$  degradation, suggesting that p38MAPK phosphorylation of ER $\alpha$  at Ser-294 is required for ER $\alpha$  ubiquitination by Skp2.

Of note, interaction of Skp2 was specific for ER $\alpha$ , not seen for ER $\beta$  or several other nuclear receptors, suggesting different mechanisms for the regulation of intracellular levels of the two ERs. Interestingly, by the use of ER $\alpha$ -AB/ ER $\beta$ -CDEF chimaera (with AB-domain of ER $\alpha$  fused to C-terminus of ER $\beta$ ) we were able to make Skp2 interact with ER $\beta$  while still not able to ubiquitinate the receptor. These findings further highlight the important role of unique primary protein sequence between the two ERs as a determinant of differential post-translational modifications on the two receptors which in turn

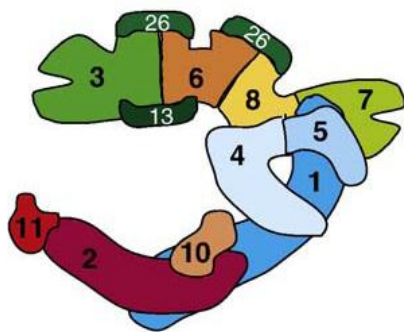
regulates very different mechanisms of their cellular turnover and functions in cells. Thus, we identify Skp2 as a new E3-ubiquitin ligase specific for ER $\alpha$ , and highlight a new ER $\alpha$  degron phospho-code involving p38MAPK dependent phosphorylation of ER $\alpha$  at Ser294.

## INTRODUCTION

In the regulation of cellular levels of proteins by the ubiquitin proteasome system, the target specificity often comes from the E-3 ubiquitin ligase. Studies on substrate recognition by E-3 ubiquitin ligases have often revealed the requirement of a specific recognition motif or post-translational modification in the sequence of the target protein. We would therefore discuss here the substrate recognition motifs for the two E-3 ubiquitin ligases that we described previously to be essential for cell cycle regulation, namely APC/C and SCF.

### ***APC/C Structure and Substrate Recognition Motifs***

The destruction of a number of APC/C substrates requires a recognition sequence called the D-box which minimally comprises the consensus sequence RXXL but may include additional primary sequence surrounding the D-box. A second motif called the KEN box (usually KENxxxE/D/N) is often required for ubiquitination and destruction of APC/C substrates after anaphase and in G1, when APC/C Cdh1 is active, whereas D-boxes can function as recognition sequences for both APC/C Cdc20 and APC/C Cdh1. However, a switch from Cdc20 to Cdh1-dependent APC/C activation cannot simply be interpreted as an acquired ability of the APC/C to recognize a functional KEN box in addition to the D-box. With an increasing number of APC/C substrates being characterized, there appears to be a remarkable variation between the primary destruction motifs of APC/C substrates.



Subunit	Predicted Mw (H.s.;kDa)	Functional Aspects
APC1/Tsg24	216	mitotic phosphosites; scaffold?
APC2	94	cullin
APC3/Cdc27	92	TPR; mitotic Phosphosites
APC4	92	connector?
APC5	85	connector?
APC6/Cdc16	72	TPR; mitotic phosphosites
APC7	63	TPR; mitotic phosphosites
APC8/Cdc23	69	TPR; mitotic phosphosites
APC10/Doc1	21	processivity factor
APC11	11	RING-E3
APC13	9	stability factor?
Cdc26	10	stability factor?

Courtesy: Van Leuken R et al. Biochem Biophys Acta. 2008 Sep; 1786(1):49-59

### **Fig.3.1 Structure and subunit composition of E3 Ubiquitin Ligase: APC/C**

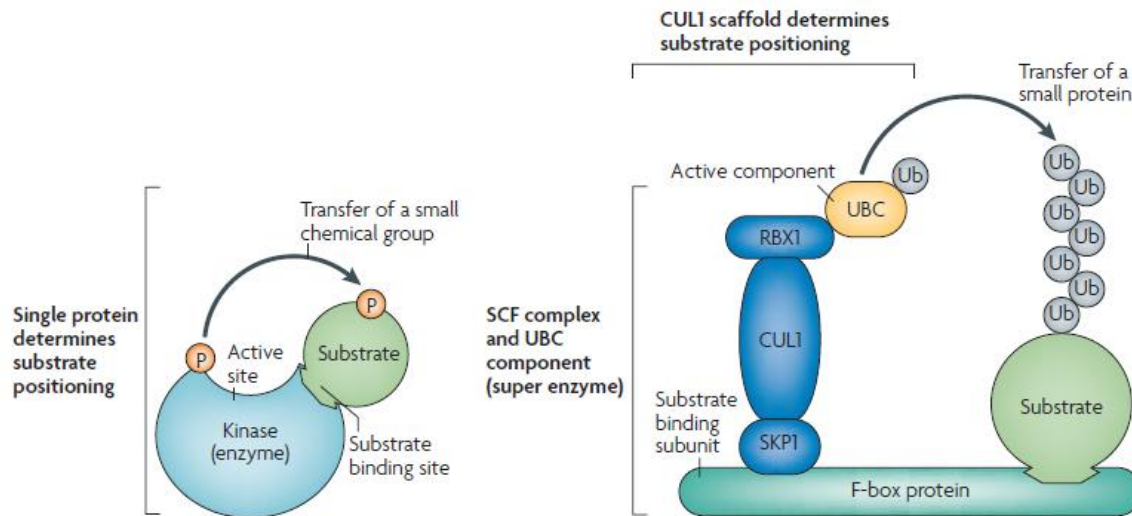
The APC/C is a huge E3 enzyme: an emerging composition of the APC/C. APC1 acts as a scaffold for two subcomplexes, a structural block and a catalytical block. The structural block contains TPR subunits APC3/CDC27, APC6/CDC16, APC7 and APC8/Cdc23 and the catalytical block contains the cullin APC2, the ring finger APC11 and the APC10/Doc1 processivity factor. APC4 and APC5 may connect the TPR block to the catalytic block. Only mammalian APC/C has the additional TPR subunit APC7, which is related to APC3/CDC27, but no data on its position within the APC/C are available. Cdc26 and APC13 may act as stability factors connecting the TPR subunits. Cdc20 may bind to the TPR subunits, but a recent model places Cdc20 close to APC2 and APC11 at the catalytic sub-complex.

For example, the region required for destruction of the APC/C Cdc20 substrate Cyclin A comprises a domain C-terminally of the D-box and APC/CCdh1-dependent destruction of Cdc20, apart from its KEN box, may require a different motif called the CRY box (CRYxPS), first identified in germinal vesicle stage mouse oocytes and mouse embryos. As another example, Aurora A has both a D-box and a KENbox, but in addition lends its name to an A-box (RxLxPSN), a destruction motif that is found in all vertebrate Aurora As, but not in Aurora B or C. Furthermore, the degradation of XKid, which has a role in chromosome segregation in anaphase, is in part dependent on APC/CCdc20 but this demands a KEN like

GxEN box. It is remarkable that functional destruction motifs often reside close to the N- or C-terminus of APC/C-substrates and D-boxes may be particularly located in protein regions that are natively unfolded. Intriguingly, multi-ubiquitination of the lysine residues of APC/C substrates occurs randomly, which fits with the idea that D-boxes may act mainly as a recognition sequence for the APC/C, whereas any lysine residue is exposable to the active site of the APC/C by random motion of the substrate. Flexibility in the region surrounding the D-box would also facilitate the addition of lengthy poly-ubiquitination tails (Leuken R et al.).

### ***SCF Structure and Substrate Recognition Motifs***

SCF complexes are super-enzymes. The SCF (SKP1–CUL1–F-box protein) ubiquitin ligase complex is the best characterized mammalian cullin RING ubiquitin ligase (CRL). The cullin subunit CUL1 functions as a molecular scaffold that interacts at the amino terminus with the adaptor subunit SKP1 (S-phase kinase-associated protein 1) and at the carboxyl terminus with a RING-finger protein RBX1 (also known as ROC1), RBX2 (also known as ROC2) or Ro52 and a specific E2 enzyme or ubiquitin conjugating enzyme (UBC), such as UBC3, UBC4 or UBC5. The F-box protein functions as the variable component that binds SKP1, through the F-box domain, and the substrate, through different protein–protein interaction motifs, which in most cases are localized C-terminally of the F-box.



Courtesy: David Frescas & Michele Pagano Nature Reviews Cancer 8, 438-449 (June 2008)

### **Fig.3.2 SCF complexes are super-enzymes**

The SCF (SKP1–CUL1–F-box protein) ubiquitin ligase complex is the best characterized mammalian cullin RING ubiquitin ligase (CRL). The cullin subunit CUL1 functions as a molecular scaffold that interacts at the amino terminus with the adaptor subunit SKP1 (S-phase kinase-associated protein 1) and at the carboxyl terminus with a RING-finger protein RBX1 (also known as ROC1), RBX2 (also known as ROC2) or Ro52 and a specific E2 enzyme or ubiquitinconjugating enzyme (UBC), such as UBC3, UBC4 or UBC5. The F-box protein functions as the variable component that binds SKP1, through the F-box domain, and the substrate, through different protein–protein interaction motifs, which in most cases are localized C-terminally of the F-box.

In many ways, the molecular composition and functionality of SCF ligases, together with the UBC component, can be considered a super-enzyme. In the classical enzymatic reaction the enzyme such as a kinase transfers a small chemical group (like, a phosphate) by way of an active catalytic site to targeted substrates. These substrates are selected on the basis of their ability to bind the specific kinase through a substrate-binding domain. The orientation of the substrate and its positioning towards the active site is determined by the sequence of the protein chain. In comparable mechanical fashion, SCF ubiquitin ligases transfer a small protein (that is, the ubiquitin moiety) by way of an activated UBC component to

specific substrates that are selected through a particular substrate-binding protein (that is, the F-box protein). Here, the cullin and F-box protein dictate the orientation of the substrate and its presentation to the RING-finger protein–UBC pair. Most SCF substrates are recognized and bound by the F-box protein subunit only when they are post-translationally modified, usually through phosphorylation at specific sites [1-4]. This is in contrast to other ligases, such as APC/C, which are only activated when needed and recognize substrates on the basis of a degradation motif (degron) in the primary sequence of their targets. Sixty-nine F-box proteins have been identified in humans (Jin J, et al.2004, Cenciarelli C, et al. 1999, Winston J.T, et al. 1999) and they have been classified into three categories: those with WD40 domains (FBXWs), those with leucine-rich repeats (FBXLs) and those with other diverse domains (FBXOs).

Notably, only nine of the sixty-nine SCF ubiquitin ligases have well-established or proposed substrates; these include SCF $\beta$ -TrCP1, SCF $\beta$ -TrCP2, SCFSKP2, SCFFBXL3, SCFFBXL20, SCFFBXO4, SCFFBXW7, SCFFBXO7 and SCFFBXW8. Substrates of these SCF complexes can be sub-divided into two main groups: direct regulators of cyclin-dependent kinases (CDKs) and regulators of gene transcription (or both) [1-4]. Numerous studies have described the roles of SCF ubiquitin ligases in controlling cell size, proliferation and survival, and, given the diverse and important roles of SCF ligases, their deregulation has been implicated in aberrant cellular growth and tumorigenesis. Such is the case for Skp2, which has a central role in cell cycle progression, cellular growth and differentiation by targeting oncogenic proteins for degradation. Since Skp2, according to our work, was shown to regulate the levels of ER $\alpha$  we investigated the role of post-translational modifications on ER $\alpha$  as an underlying mechanism for this regulation.

## EXPERIMENTAL PROCEDURES

### ***Antibodies-***

Anti-Skp2 (N-19, H-435, A-2), anti-ER $\alpha$  (HC-20, F-10), anti-Ub (P4D1), anti-HA tag (F-7, Y-11), anti-GST (Z-5) were purchased from Santa Cruz Biotechnology. Anti-Myc tag (2272, 2276), anti-p38MAPK (9212, 9228), anti-phospho (Thr180/Tyr182) p38MAPK (9211, 9216), anti-ATF2 and anti-phospho ATF-2 were from Cell Signaling, anti-flag rabbit and mouse from Sigma and anti-phosphoserine (ab17465) were purchased from Abcam. Horseradish peroxidase (HRP)-conjugated donkey anti-goat, donkey anti-mouse and donkey anti-rabbit IgG secondary antibodies were from Santa Cruz Biotechnology.

### ***Cell Culture and Transfections-***

Cos-1 cells were purchased from American Type Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 units per ml of Penicillin and 50  $\mu$ g/ml of Streptomycin at 37°C under 5%CO<sub>2</sub> in humidified air. MCF-7 cells were maintained in growth medium containing Minimum Essential Medium (MEM)-with phenol red supplemented with 5% heat inactivated Calf Serum (CS), 100 units per ml of Penicillin and 50  $\mu$ g/ml of Streptomycin at 37°C in non-CO<sub>2</sub> incubator or treatment medium containing Minimum Essential Medium (MEM)-without phenol red supplemented with 5% Charcoal dextran stripped Calf Serum (CS) at 37°C under 5%CO<sub>2</sub> in humidified air. ZR-75 cells were maintained in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 100 units per ml of Penicillin and 50  $\mu$ g/ml of Streptomycin at 37°C and 5%CO<sub>2</sub> in humidified air. All transient plasmid transfections were performed using LipofectAMINETM 2000 Reagent from Invitrogen according to manufacturer's instructions. siSkp2 transfection was performed using Lipitoid reagent kindly provided by Dr.Ronald Zuckermann at the Molecular Foundry, Lawrence Berkeley National Laboratory (23). All other siRNA transfections were performed using Dharmafect transfection reagent from Dharmacon.

### ***Plasmids-***

Skp2 cDNA was purchased by Open Biosystems and cloned into pcDNA3-Flag expression vector. Various Skp2 deletion mutants were generated from this full-length construct by PCR based subcloning. ER $\alpha$  was cloned into either pcDNA3-Flag expression vector or pcDNA3.1-Myc/His expression vector from Invitrogen. Various ER $\alpha$  deletion mutants were generated from the full-length Myc tagged construct by PCR based subcloning. Site directed mutagenesis kit from Stratagene was used to introduce various Ser to Ala or Glu and Lys to Ala or Arg point mutations in pcDNA3-Flag-ER $\alpha$  (full-length). pCMV-ER $\alpha$ / $\beta$  chimeras were generated previously in our laboratory (Sheng et al.). pCMV-HA-Ubiquitin was generated by subcloning from GST-Ubiquitin expression vector (Addgene plasmid 10861) originally generated by Peter Howley. Stub1/CHIP donor plasmid was obtained from plasmID-repository at Harvard (HIP Clone ID# 194919) and later subcloned into pcDNA3.1-Myc/His expression vector before use. pMEV-2HA-p38MAPK-WT (wildtype) and DN (dominant negative) mutant were purchased from Biomyx Technology. Myc-tagged Cul1, Cul2, Cul3, Cul4, Cul5, Cul7 and HA-tagged Rbx1 and Rbx2 were generated by subcloning from cDNA vectors obtained from Addgene. Adenoviral vectors encoding Skp2-WT or  $\Delta$ F-box mutant were constructed by cloning relevant sequences into pAdTrack vector purchased from Stratagene. Details for generation of any of the above mentioned expression vector, is available upon request.

### ***Silencing by Small Interfering RNA (siRNA)-***

MCF-7 or ZR-75 cells seeded onto 6-well plates at a confluence of 40-60% were transfected with siRNA duplexes, siSkp2 (Qiagen) or sip38MAPK (Dharmacon) or non-targeting control siGL-3 (Dharmacon or Qiagen). siSkp2 transfection was performed using Lipitoid reagent kindly provided by Dr. Ronald Zuckermann at the Molecular Foundry, Lawrence Berkeley National Laboratory (23, 24). All other siRNA transfections were performed using Dharmafect transfection reagent from Dharmacon.



### ***Adenovirus Construction and Infection for Over-expression of Endogenous Factors-***

cDNAs for the genes of interest, namely Skp2-WT, Skp2 $\Delta$ Fbox, p38MAPK-WT, p38MAPK-DN (dominant negative) and ER $\alpha$  were first cloned into pAdTrack-CMV shuttle vector from Stratagene using PCR based subcloning. DNA from positive clones was linearized by digestion with PmeI and electroporated into electrocompetent BJ5183 cells (Stratagene) already containing plasmidP1 for the recombination event to occur. Recombined DNA was thereafter extracted and transfected into AD293 cells for adenovirus preparation. After rounds of amplification the virus obtained was then purified by CsCl density gradient centrifugation and characterized for its titre and ability to over-express the protein of interest. MCF-7 cells seeded onto 6-well plates at a confluence of 40-60% were thereafter infected with this virus or an equal MOI (Multiplicity of Infection) of a non target control virus followed by ligand treatment (if required). Cells were harvested and processed for RNA and/or protein extraction as per requirement.

### ***Co-Immunoprecipitation assay-***

Cos-1 cells were seeded onto 6-well plates at a confluence of 80-90% and transfected with plasmids of interest. 24h post transfection cells were harvested, washed with ice-cold PBS and solubilized with 0.5ml CoIP buffer (50mM Tris pH7.5, 150mM NaCl, 0.1% Nonidet P-40, 5mM EDTA, 0.1mM sodium orthovanadate, 1mM dithiothreitol (DTT), 1mM PMSF and 1X Protease inhibitor cocktail from Roche). Immunoprecipitation was carried out using 2-5ug specific antibody by incubation at 4°C overnight with gentle rotation. The precipitated proteins were collected by incubating with Protein-G sepharose (GE Healthcare) for 2h at 4°C with gentle rotation. After low speed centrifugation to remove the supernatant, the agarose was washed with CoIP buffer, boiled in SDS sample buffer and subjected to SDS-PAGE and Western blotting. In case of MCF-7 cells, endogenous proteins were Immunoprecipitated from lysates obtained from 10cm<sup>2</sup> plates using similar protocol as mentioned above without the involvement of transfections or adenoviral infections for exogenous over-expression of factors.

### ***Ubiquitination assay-***

Cos-1 cells transfected with ER $\alpha$  and other plasmids were pretreated with 10 $\mu$ M MG-132 (Calbiochem, La Jolla, CA) for 5h to block proteasome activity, before harvesting them 24h post transfection. Following harvest, cells were lysed in buffer containing 50mM Tris pH 7.5, 150mM NaCl, 10% glycerol, 5mM EDTA, 1% Nonidet P-40, 0.1mM sodium orthovanadate, 1mM dithiothreitol (DTT), 1mM PMSF and 1X Protease inhibitor cocktail from Roche. Immunoprecipitation was carried using 3 $\mu$ g ER $\alpha$  antibody by incubation at 4°C overnight with gentle rotation. The precipitated proteins were collected by incubating with Protein-G sepharose (GE Healthcare) for 2h at 4°C with gentle rotation. After low speed centrifugation to remove the supernatant, the agarose was washed with CoIP buffer, boiled in SDS sample buffer and subjected to SDS-PAGE and Western blotting with anti-HA antibody (Santa Cruz). In case of MCF-7 cells, endogenous ER $\alpha$  protein was Immunoprecipitated from lysates obtained from 15cm<sup>2</sup> plates following 48h over-expression of either Adeno- $\beta$ -gal or Adeno-Skp2 or Adeno-p38MAPK and 5h treatment with 10 $\mu$ M MG-132 (Calbiochem, La Jolla, CA) to block proteasome activity, using similar protocol as mentioned above and ubiquitinated ER $\alpha$  was detected by anti-Ubiquitin antibody.

### ***In Vitro Kinase assay-***

To perform in vitro kinase assay, 1 $\mu$ g of recombinant active phospho-GST-p38MAPK or inactive GST-p38MAPK protein (Invitrogen) was incubated with 1 $\mu$ l of in vitro translated ER $\alpha$ -WT or ER $\alpha$ -S294A protein as substrate, in the presence of 1X Kinase buffer (25mM Tris-HCl (pH 7.5), 5mM beta-glycerophosphate, 2mM dithiothreitol (DTT), 0.1mM Na<sub>3</sub>VO<sub>4</sub>, 10mM MgCl<sub>2</sub> (Cell Signaling Technology) supplemented with 200 $\mu$ M ATP in a total reaction volume of 50 $\mu$ l. The reactions were incubated at 30°C for 30min for enzymatic catalysis to occur and thereafter terminated by addition of 3X SDS sample buffer and subjected to western blotting.

### ***In Cell Kinase assay-***

To perform in cell kinase assay, Cos-1 cells were transfected with Flag-Skp2-WT or Flag-Skp2-S64A alone or along with HA-p38MAPK-WT or dominant negative kinase dead HA-p38MAPK-DN mutant with either no treatment or treatment with vehicle (ethanol), E2 (1nM) or tamoxifen (1 $\mu$ M) 6h post transfection. Cells were harvested 24h post ligand treatment and subjected to immunoprecipitation in CoIP buffer, as described earlier, using phosphoserine-antibody conjugated agarose beads (Sigma) followed by SDS-PAGE separation and western blot analysis using Skp2 and p38MAPK antibodies. Phosphorylation event was detected by an upward shift of the Skp2 band in the presence of the wildtype kinase, not the kinase dead mutant.

## **RESULTS AND EXPERIMENTAL FINDINGS**

### ***Phosphorylation at Serine-294 in ER $\alpha$ is Required for Its Ubiquitination by Skp2***

Skp2 is a member of the FBL (L for Leucine Rich Repeat-LRR) subclass of F-box proteins that require phosphorylation signals on their target proteins as part of the recognition process for their ubiquitination. We investigated this specific phospho-code that appears to be essential for the ubiquitination of the target proteins for ER $\alpha$  by generating multiple serine point mutants of the receptor, previously characterized to be important for its biological functions in these cells. We then performed ubiquitination assays for ER $\alpha$ -WT (wildtype) or various Serine point mutants in the presence or absence of Skp2 and found an increase in ER $\alpha$  ubiquitination on over-expressing Skp2 for all but ER $\alpha$ -S294A mutant (Figure 3.3a) thereby suggesting Serine-294 in ER $\alpha$  to be essential for its ubiquitination by Skp2. To confirm this further we performed over-expression of Skp2 in Cos-1 cells in the presence of either ER $\alpha$ -WT (wildtype) or ER $\alpha$ -S294A and found a dose dependent decrease in the level of ER $\alpha$ -WT protein with over-expression of Skp2 (which could be reversed by proteasome inhibitor MG-132)

whereas ER $\alpha$ -S294A mutant remained resistant to Skp2 mediated degradation (Figure 3.3b). Of note the S294E mutant which had the serine residue replaced with a glutamic acid that acts as a phospho-mimic showed enhanced ubiquitination in the presence of over-expressed Skp2 just like the wildtype receptor (Figure 3.4a) thereby indicating phosphorylation of ER $\alpha$  at Ser-294 as absolutely required for Skp2 mediated turnover of the receptor.

Since Stub1 is another known E3-ligase for ER $\alpha$  that is known to ubiquitinate the receptor in a hormone independent manner also, we wanted to see if it also requires the same phospho-code on ER $\alpha$  as seen for Skp2 (25). However our data suggests Ser-294 phosphorylation on ER $\alpha$  to be a mechanism of regulation rather specific to Skp2 as Stub1 was able to ubiquitinate both wildtype and S294A mutant equally efficiently, failing to distinguish between the two forms of the receptor, unlike Skp2 (Figure 3.4b). We next performed sequence alignment of Serine-294 containing peptide in ER $\alpha$  protein from various species and found the entire 10 amino-acid sequence, esp. the Serine-294 residue, to be highly conserved across various species thereby highlighting the importance of regulation of ER $\alpha$  levels and function through this residue (Figure 3.4c). Having identified Skp2 as a novel E3-ubiquitin ligase for ER $\alpha$ , we next wanted to investigate if this regulation via proteasome mediated degradation by Skp2 was specific to ER $\alpha$ . We therefore checked a close counterpart Estrogen Receptor-beta (ER $\beta$ ) and a few other nuclear receptors and transcription factors like Thyroid Receptor (TR), Retinoic Acid Receptor (RAR $\alpha$ ) and cAMP Response Element Binding protein (CREB). Our data suggest the interaction of Skp2 with ER $\alpha$  to be highly specific as we could not detect interaction between Skp2 with any of the mentioned factors closely related in function to ER $\alpha$  (Figure 3.5a).

Since ER $\alpha$  and ER $\beta$  proteins share high sequence homology with the most variable domain being the AB-domain (previously shown by us to be essential for interaction with Skp2), we investigated if swapping the AB-domains between the two ER proteins could alter their ability to interact with Skp2. To this end we used two ER $\alpha$ /ER $\beta$  chimaeras- first with AB-domain of ER $\alpha$  and CDEF-domain of ER $\beta$  (ER $\alpha$ -

AB/ER $\beta$ -CDEF) and other with AB-domain of ER $\beta$  and CDEF-domain of ER $\alpha$  (ER $\beta$ -AB/ER $\alpha$ -CDEF). We indeed found that just by replacing their AB domains with that of the other we were able to alter their ability to interact with Skp2, as the chimaera with ER $\alpha$ -AB/ER $\beta$ -CDEF could interact with Skp2 unlike ER $\beta$  and the one with ER $\beta$ -AB/ER $\alpha$ -CDEF could no more interact with Skp2 unlike ER $\alpha$  (Figure 3.5b). Intrigued by this we wanted to further investigate if transfer of interaction function to ER $\alpha$ -AB/ER $\beta$ -CDEF chimaera would also render it as a target for Skp2 mediated ubiquitination. However, our data showed that replacement of AB-domain of ER $\beta$  by that of ER $\alpha$  could not make ER $\beta$  get ubiquitinated by Skp2 (Figure 3.5c) thereby suggesting that Skp2 mediated degradation of ER $\alpha$  is a two step process whereby first step is interaction between the two proteins and next is ubiquitination of ER $\alpha$  which probably requires very precise post translational modification(s) of ER $\alpha$  protein on residues which probably are not conserved between ER $\alpha$  and ER $\beta$ . To establish this hypothesis we performed sequence alignment of ER $\alpha$  and ER $\beta$  proteins and found Serine-294 residue in ER $\alpha$  to be not conserved in ER $\beta$ , thereby explaining the inability of ER $\alpha$ -AB/ER $\beta$ -CDEF chimaera to be ubiquitinated by Skp2 inspite of its successful interaction with the ubiquitin ligase (Figure 3.6). This suggests very different mechanisms that regulate the two ERs at the protein level most likely by involvement of unique ubiquitin-proteasome machinery. We thus, propose Ser-294 phosphorylation as a precise and specific phospho-degron code on ER $\alpha$  for its ubiquitination and hence degradation by Skp2.

### ***p38MAPK Mediated Phosphorylation of ER $\alpha$ at Serine-294 is Required for its Ubiquitination by Skp2***

After establishing the importance of phosphorylation of ER $\alpha$  at Ser-294 for its degradation by Skp2, we next wanted to investigate the kinase involved in regulating the degradation of ER $\alpha$  through Skp2 by phosphorylating ER $\alpha$  at this particular serine residue. Bioinformatic analysis of ER $\alpha$  protein sequence revealed the peptide sequence containing Ser-294 residue as a putative Cdk2 or MAPK-family

target site. To address the possibility of the role of either of the two kinases in regulating Skp2 mediated ER $\alpha$  turnover, we performed ubiquitination assay for ER $\alpha$  in the absence or presence of over-expressed Skp2 in Cos-1 cells (as described earlier), this time also in the absence or presence of specific inhibitors to various Cdk-family, MAPK-family, PI3K, Protein Kinase A, B, C, JNK/SAPK and p38MAPK. Data shown for Cdk-2 and p38MAPK inhibitors (Figure 3.7a). Only in the presence of a specific p38MAPK inhibitor were we able to prevent Skp2 mediated ubiquitination of ER $\alpha$  indicating the importance of this kinase in the regulation of Skp2 mediated turnover of the receptor. To confirm this we over-expressed either wildtype p38MAPK-WT or dominant negative p38MAPK-DN mutant in Cos-1 cells and then performed ubiquitination assay for ER $\alpha$  as above. We found a dose dependent increase in Skp2 mediated ER $\alpha$  ubiquitination on over-expressing wildtype p38MAPK which was blocked by the dominant negative mutant thereby suggesting the phosphorylation of ER $\alpha$  by p38MAPK as an essential step for its ubiquitination by Skp2 (Figure 3.8a). Of note, ER $\alpha$  interaction was equally well for both wildtype and mutant kinase thereby suggesting that blockage of Skp2 mediated ubiquitination in the presence of dominant negative p38MAPK was purely because of inability of the mutant to phosphorylate ER $\alpha$ .

We then performed an in vitro kinase assay to check if p38MAPK was phosphorylating ER $\alpha$  at Ser-294 residue to regulate its degradation by Skp2. We performed in vitro phosphorylation of ER $\alpha$ -WT or ER $\alpha$ -S294A in the presence of either active p38MAPK-WT or the dominant negative mutant and detected phosphorylation of ER $\alpha$ -WT but not the S294A mutant specifically in the presence of active p38MAPK (Figure 3.8c) thereby suggesting Ser-294 in ER $\alpha$  to be a p38MAPK target site. Previous studies have shown that p38MAPK exists in complex with ER $\alpha$  in breast cancer cells so we wanted to check what regions of ER $\alpha$  protein interact with p38MAPK. For this we performed co-immunoprecipitation assays with wildtype p38MAPK and various deletion mutants of ER $\alpha$ . Our results indicate the p38MAPK binds to the N-terminal region of ER $\alpha$  (data not shown). We next wanted to check if Skp2 was in complex with p38MAPK and hence performed co-immunoprecipitation assays with Skp2 and either p38MAPK-WT or

p38MAPK-DN and found that both the wildtype and dominant negative mutant forms of p38MAPK exist in complex with Skp2, as seen for ER $\alpha$  (data not shown).

To establish the occurrence of this phenomenon in breast cancer cells we performed ubiquitination assay for ER $\alpha$  in the absence or presence of over-expressed p38MAPK through infection of MCF-7 cells with Adenoviral-p38MAPK. Following immunoprecipitation for ER $\alpha$  and detection of ubiquitinated form of the receptor using anti-Ubiquitin antibody we saw significant increase in ubiquitinated ER $\alpha$  in the presence of over-expressed p38MAPK compared to its absence, suggesting involvement of p38MAPK in the ubiquitination of ER $\alpha$  in these cells (Figure 3.7b). Over-expression of Skp2 in breast cancer cells in the presence of p38MAPK inhibitor was ineffective in causing ER $\alpha$  degradation (Figure 3.8b) thereby suggesting a role of signaling through p38MAPK in regulating the turnover of the nuclear receptor. We also have preliminary evidence of a possible role of this regulation in vivo as our immunohistochemical analysis on primary breast tumors from human subjects showed increased staining for phosphorylated p38MAPK in ER $\alpha$ -negative tumors, being also high in Skp2 levels, compared to ER $\alpha$ -positive, which we believe may underlie the reason for diminished receptor levels in these breast tumors (Figure 3.9a). We also checked p38MAPK expression in various ER $\alpha$ -positive and negative breast cancer cell-lines and found p38MAPK to be expressed at higher levels in cells that did not express ER $\alpha$ , a phenomenon very similar to Skp2, described previously in Figure 2.7b (Figure 3.9b).

In order to implicate p38MAPK for the absence of ER $\alpha$  protein in these ER-negative cell-lines, we next wanted to check if we could restore ER $\alpha$  protein by treating these cells with either p38MAPK inhibitor and/or proteasomal inhibitor MG-132. We were indeed fascinated to observe the recovery of ER $\alpha$  protein in these ER-negative cells in the presence of either p38MAPK or proteasomal inhibitor alone or the combination, most likely due to a block in the p38MAPK initiated and Skp2 mediated proteasomal degradation of ER $\alpha$  (Figure 3.9c). We next compared the level of re-expressed ER $\alpha$  protein in these ER-negative cells to known amounts a protein in MCF-7 cells, a gold standard ER-positive breast cancer cell-

line used in much of our work and found a recovery of up to one-third of receptor levels in MDA-MB-453 and nearly half in MDA-MB-468 cells after 72h of p38MAPK inhibitor alone (Figure 3.9d). Thus, we propose phosphorylation of ER $\alpha$  at Ser-294 residue by p38MAPK as a novel phospho-degron code regulating the proteasome mediated turnover of ER $\alpha$  by Skp2 and a very promising pathway for drug targeting to restore ER $\alpha$  protein in ER-negative breast tumors in order to make them amenable to anti-estrogen therapy.

## DISCUSSION

The ubiquitin-proteasome pathway that determines abundance of regulatory proteins in cells (Hershko and Ciechanover, 1998) is under tight control through cellular signals that culminate into post-translational modifications on the substrate degrons. Given that many mutations in ER $\alpha$  protein associated with cancer affect its stability (Qun Zhou et al., 2009), characterization of the mechanism of ER $\alpha$  ubiquitination, by identifying the hotspots of protein modifications, is critical for understanding the oncogenic transformation it mediates. Steroid hormone receptors like ER $\alpha$  while activated by their cognate sex steroids, are also regulated by non-steroidal stimuli like kinase activators, phosphatase inhibitors, growth factors etc. Consistent with this, several studies have reported the phosphorylation of ER $\alpha$  by various kinases such as MAPK (Kato S et al., 1995; Bunone G et al., 1996), c-Src (Migliaccio A et al., 1996), PKA (Chen D et al., 1999), CyclinA-Cdk2 (Rogatsky I et al., 1999) and p38MAPK (Lee H et al., 2002), that are known to regulate its protein stability, transactivation function or localization in cells. We therefore investigated post-translational modifications with known and/or unknown function, as a signal for ER $\alpha$  protein ubiquitination by Skp2. We here report a novel function of phosphorylation of ER $\alpha$  at serine-294, a site with unidentified function that was recently reported to be phosphorylated in MCF-7 breast cancer cells (Atsriku C et al., 2009), in the ubiquitination of the receptor by Skp2.



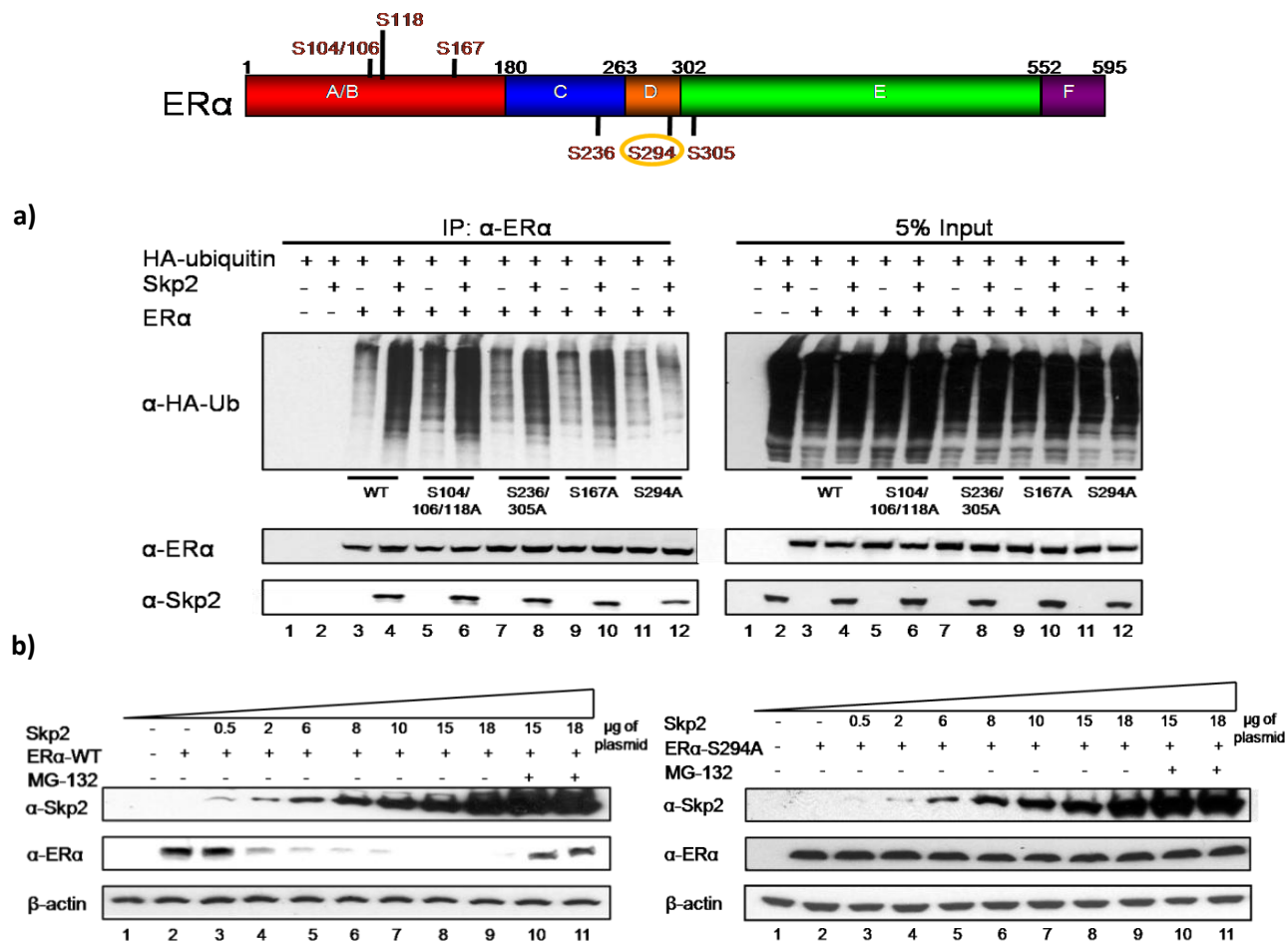
In vitro studies revealed phosphorylation of serine-294 in ER $\alpha$  by p38MAPK, the validation of which in vivo awaits availability of a phospho-specific antibody to this site and may prove to be clinically relevant. However, the importance of p38MAPK mediated phosphorylation of ER $\alpha$  in its ubiquitination by Skp2 could be validated in these cells using specific p38MAPK inhibitor and dominant negative p38MAPK. Since this site was shown to be phosphorylated both in the presence and absence of estradiol, this supports the hypothesis of its role in the ubiquitination of ER $\alpha$  by Skp2, independent of ligand. Notably, phosphorylation of Skp2 at serine-64 appeared essential for its interaction with and ubiquitination of ER $\alpha$ . Interestingly, serine-64 in Skp2 which has been suggested by previous reports as a Cdk2 phosphorylation site (Rodier G et al., 2008) was in addition identified as a p38MAPK target site, according to both mass spectrometry and in cell kinase assay (unpublished observation by Bhatt S and Katzenellenbogen BS). Our work therefore presents an interesting phenomenon where a kinase, like p38MAPK, activates both substrate (ER $\alpha$ ) as well as enzyme (Skp2) for ER $\alpha$  to undergo Skp2 mediated ubiquitination and turnover in cells.

Although phosphorylation of ER $\alpha$  at serine-294 appeared indispensable for its ubiquitination by Skp2, it was not required for the interaction of the two proteins as suggested by the interaction of ER $\alpha$ -S294A mutant with Skp2, which appeared as potent as the wildtype receptor. Interestingly, this serine residue is not conserved between ER $\alpha$  and ER $\beta$  which suggested something else unique about specificity of ER $\alpha$  interaction with Skp2, over the close homologue ER $\beta$ . Interestingly, we regained interaction between Skp2 and ER $\beta$  upon swapping ER $\alpha$ -AB domain to the C-terminus of ER $\beta$ , thereby implicating the N-terminus of ER $\alpha$  protein, known to be least conserved with ER $\beta$ , as the major determinant of its specificity of interaction with Skp2. Much to our fascination however, we did not see enhanced ubiquitination of ER $\alpha$ -AB/ER $\beta$ -CDEF-chimaera upon over-expression of the ubiquitin ligase. This suggests that ubiquitination of ER $\alpha$  by Skp2 occurs through two distinct and independent steps, first the interaction of the nuclear receptor with the ubiquitin ligase, that appears to be regulated through

phosphorylation of Skp2 at Serine-64 by p38MAPK (Chapter-IV, Fig.S1), and second its ubiquitination that apparently requires the phosphorylation of ER $\alpha$  at serine-294 by p38MAPK. Taken together these data highlight the importance of post-translational modifications in the unique regulation of proteins with otherwise high sequence homology.

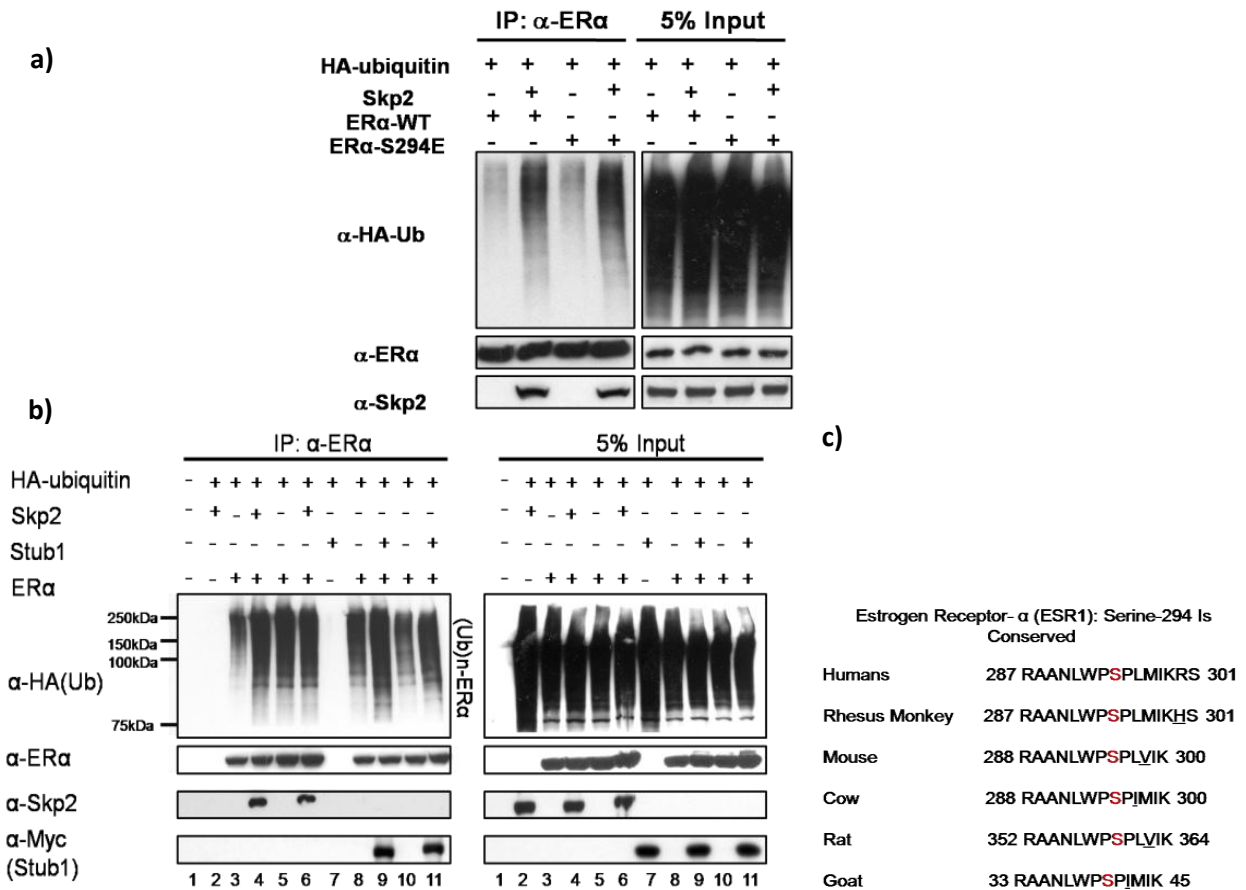
The relevance of our findings on Skp2 and p38MAPK mediated turnover of ER $\alpha$  was confirmed in primary breast tumors from human patients as immuno-histochemical analysis showed higher expression levels of both Skp2 and phospho-p38MAPK (active kinase) in ER-negative breast tumors compared to ER-positive. Although establishment of the role of Skp2 and p38MAPK mediated ER $\alpha$  turnover as a key mechanism for its regulation in vivo (in human breast tumors) needs validation in a large cohort of patients we found our preliminary findings to be in line with our in vitro data. Indeed, we observed that the treatment of ER-negative breast cancer cells (MDA-MB-453 and MDA-MB-468) with either p38MAPK inhibitor or proteasomal inhibitor MG-132 alone or in combination restored ER $\alpha$  protein levels, an observation that may have far reaching therapeutic significance. Thus our results offer a molecular mechanism for the loss of ER $\alpha$ , the target for breast cancer therapeutics, which has been observed in many clinical breast tumor samples and is correlated with aggressive malignancy and poor diagnosis. Our findings reveal a novel ER $\alpha$  phospho-degron code, involving phosphorylation by p38MAPK at Serine-294 and implicating this to the turnover of the receptor by Skp2. These novel findings hold great promise for drug targeting to restore ER $\alpha$  protein in ER-negative breast tumors in order to make them amenable to anti-estrogen therapy.

FIGURES



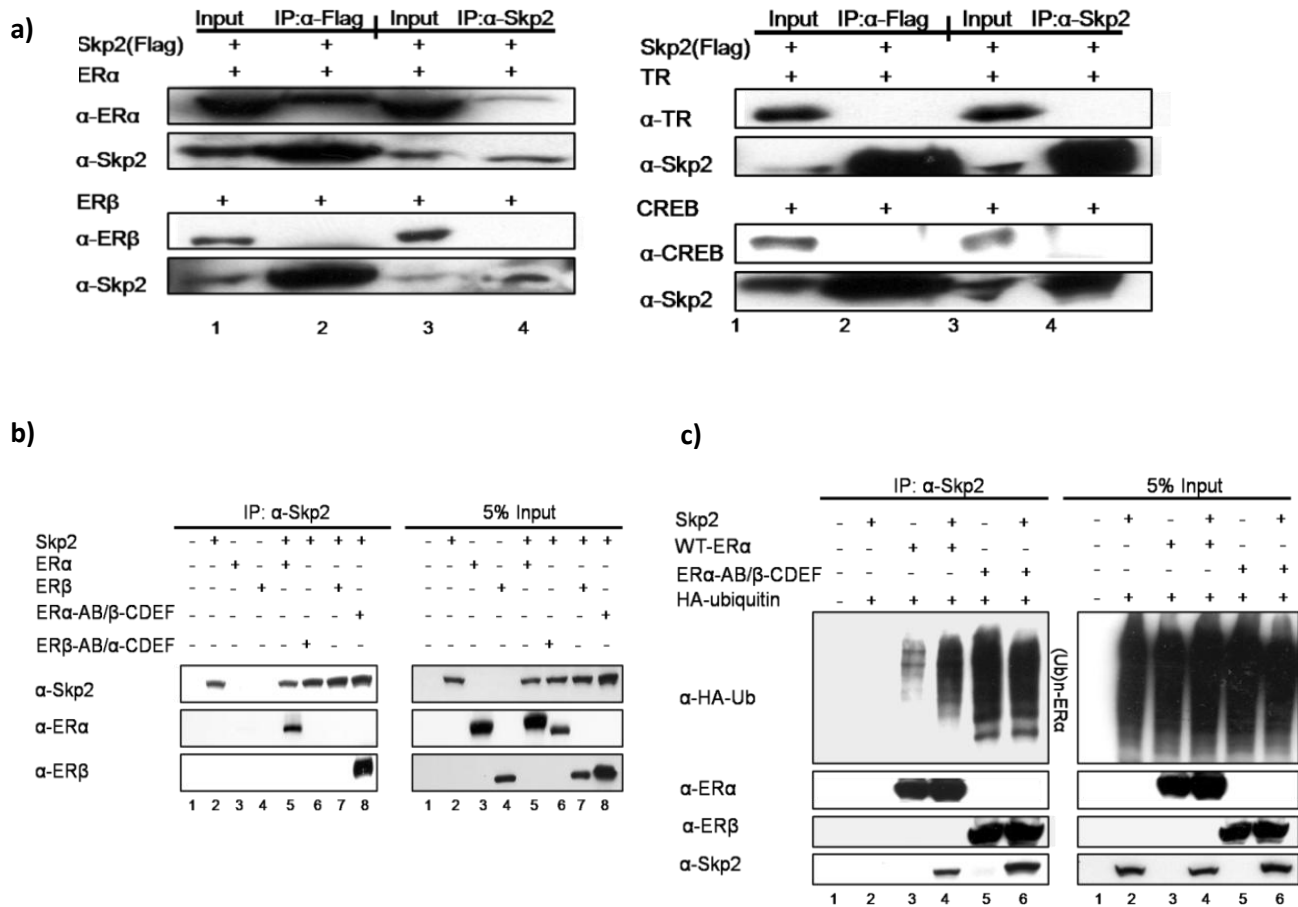
**Fig.3.3 Phosphorylation of ERα at Serine-294 is Required for Its Ubiquitination by Skp2**

a) Cos-1 cells cultured in serum free DMEM media for two days and transfected with expression plasmids for Skp2, HA-Ubiquitin along with either WT-ERα or various Serine point mutants were treated with MG-132 (10uM) for 5h in presence of serum-containing media before harvest, 24h post transfection. Cell lysates were subjected to immunoprecipitation using anti-ERα antibody followed by SDS-PAGE and western blotting for Skp2, ERα and HA. b) Cos-1 cells were transfected with increasing amounts of pCMV-Skp2 along with constant amounts of pCMV-ERα-WT or pCMV-ERα-S294A, treated with MG-132 (10uM) or not for 5h before harvest and lysed 24h post transfection for SDS-PAGE and western blot analysis.



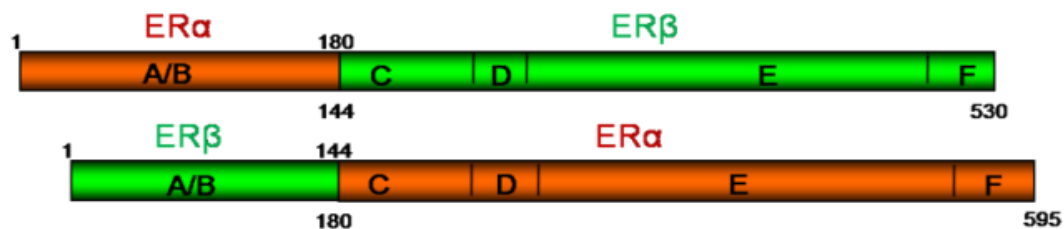
**Fig.3.4 Phosphorylation of ER $\alpha$  at Serine-294 is Required for Its Ubiquitination by Skp2**

a) Cos-1 cells cultured in serum free media for two days and transfected with expression plasmids for Skp2, HA-Ubiquitin along with either ER $\alpha$ -WT (wildtype) or ER $\alpha$ -S294E mutant were treated with MG-132 (10uM) for 5h in the presence of serum containing media before harvest, 24h post transfection. Cell lysates were subjected to immunoprecipitation using anti-ER $\alpha$  antibody followed by SDS-PAGE and western blotting for Skp2, ER $\alpha$  and HA. b) Cos-1 cells transfected with expression plasmids for Skp2, Stub1-Myc, ER $\alpha$ -WT or ER $\alpha$ -S294A mutant and HA-Ubiquitin were treated with MG-132 (10uM) for 5h before harvest, 24h-post transfection. Cell lysates were subjected to immunoprecipitation using anti-ER $\alpha$  followed by SDS-PAGE and western blotting for Skp2, Myc, ER $\alpha$  and HA. c) Sequence alignment for Serine-294 containing peptide in ER $\alpha$  from various species to show the high degree of conservation of the peptide especially Ser-294 residue across various species.



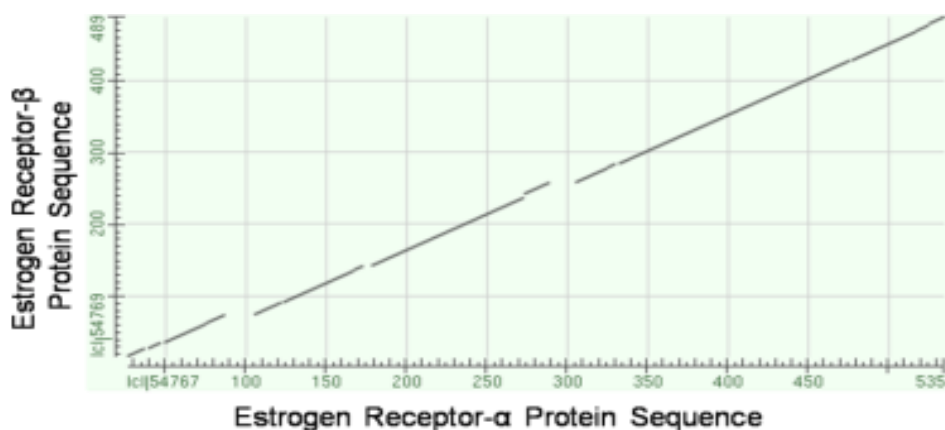
**Fig.3.5 Skp2 Mediated Ubiquitination and Regulation of Cellular Levels Is Specific for ERα and Not ERβ**

a) Cos-1 cells transfected with Flag-Skp2 along with ERα, ERβ, Thyroid Receptor (TR) or cAMP Response Element Binding Protein (CREB) were lysed 24h post transfection and subjected to immunoprecipitation using anti-Skp2 antibody followed by SDS-PAGE and western blotting with anti-Skp2, anti-ERα, anti-ERβ, anti-GR and anti-CREB. b) Chimaera Co-IP. Cos-1 cells transfected with expression plasmids for Skp2 along with WT-ERα, WT-ERβ, ERα-AB/ERβ-CDEF, ERβ-AB/ERα-CDEF were lysed 24h post transfection and subjected to immunoprecipitation using anti-Skp2 antibody followed by SDS-PAGE and western blotting with anti-Skp2, anti-ERα, anti-ERβ. c) Chimaera Ubiquitination assay. Cos-1 cells transfected with Skp2, HA-Ubiquitin expression vectors along with WT-ERα or ERα-AB/ERβ-CDEF were lysed 24h post transfection and subjected to immunoprecipitation using anti-ERα (H226) antibody followed by SDS-PAGE and western blotting with anti-Skp2, anti-ERα and HA.



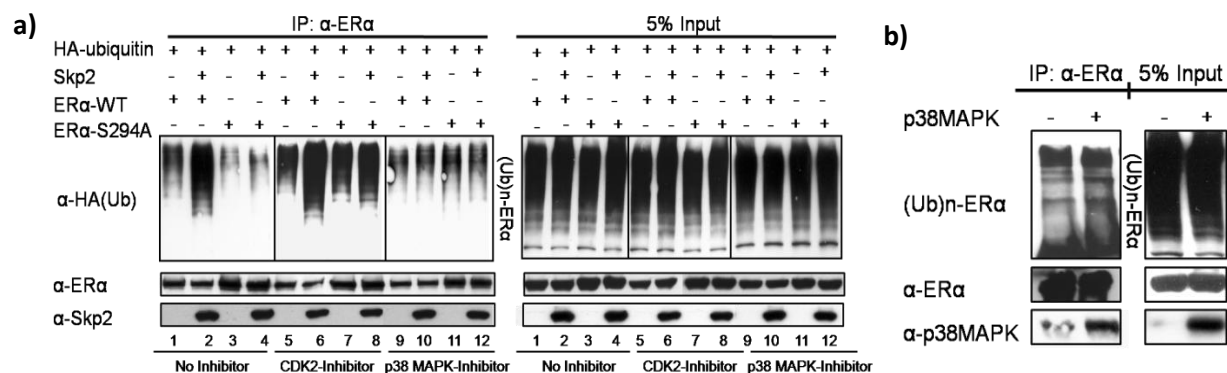
Serine-294 in ERα is NOT conserved in ERβ

ERα	274	GEGRGEVGSAGDMRAANLWP	<b>S</b> *	PLMIKRSKKNSLALSLTADQMVSAL	319
ERβ	243	GKAKRSGGHAPRVRE	L	-----LLDAL <del>S</del> PEQLVLT	272



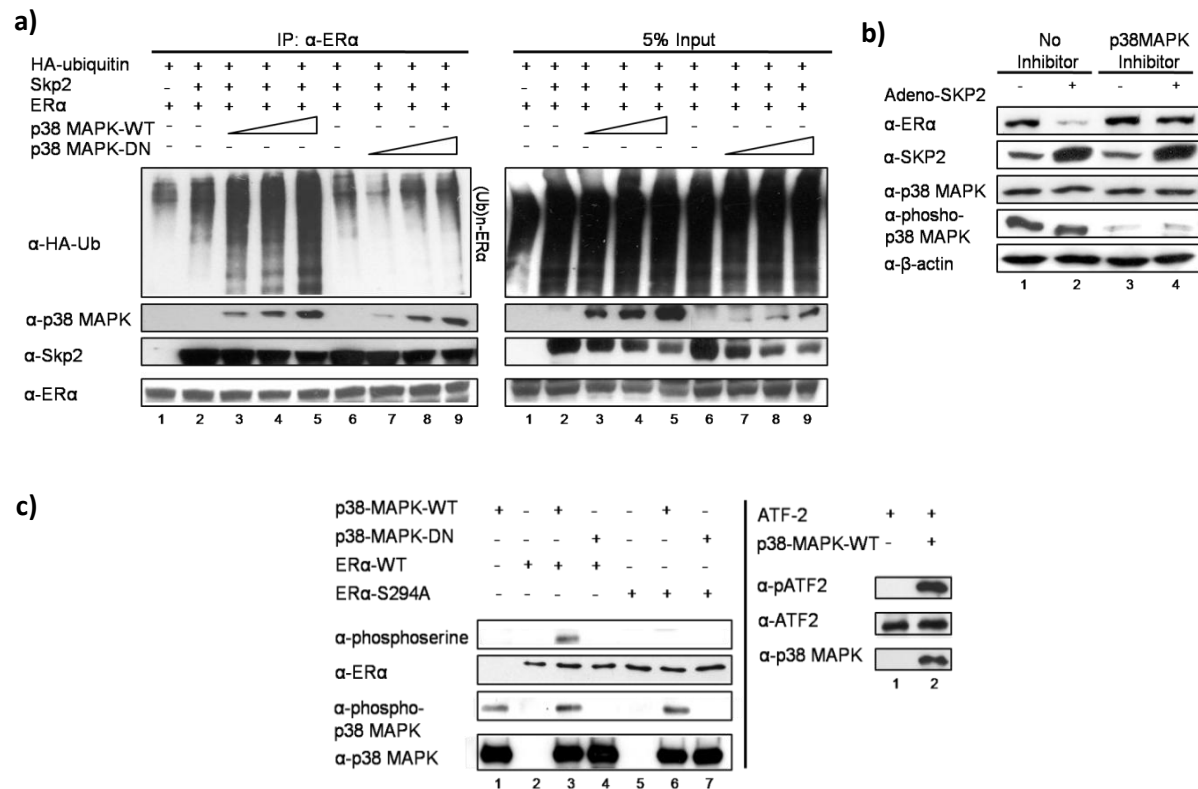
**Fig.3.6 Skp2 Mediated Ubiquitination Is Specific for ERα and Not Seen for ERβ as Ser-294 Residue is Not Conserved Between the Two ERs**

Sequence alignment of ERα and ERβ proteins using NCBI-Blast showing the non-conserved Serine-294 residue between the two receptors, being present in ERα but absent from ERβ



**Fig.3.7 p38MAPK Over-expression Causes Increased ER $\alpha$  Ubiquitination**

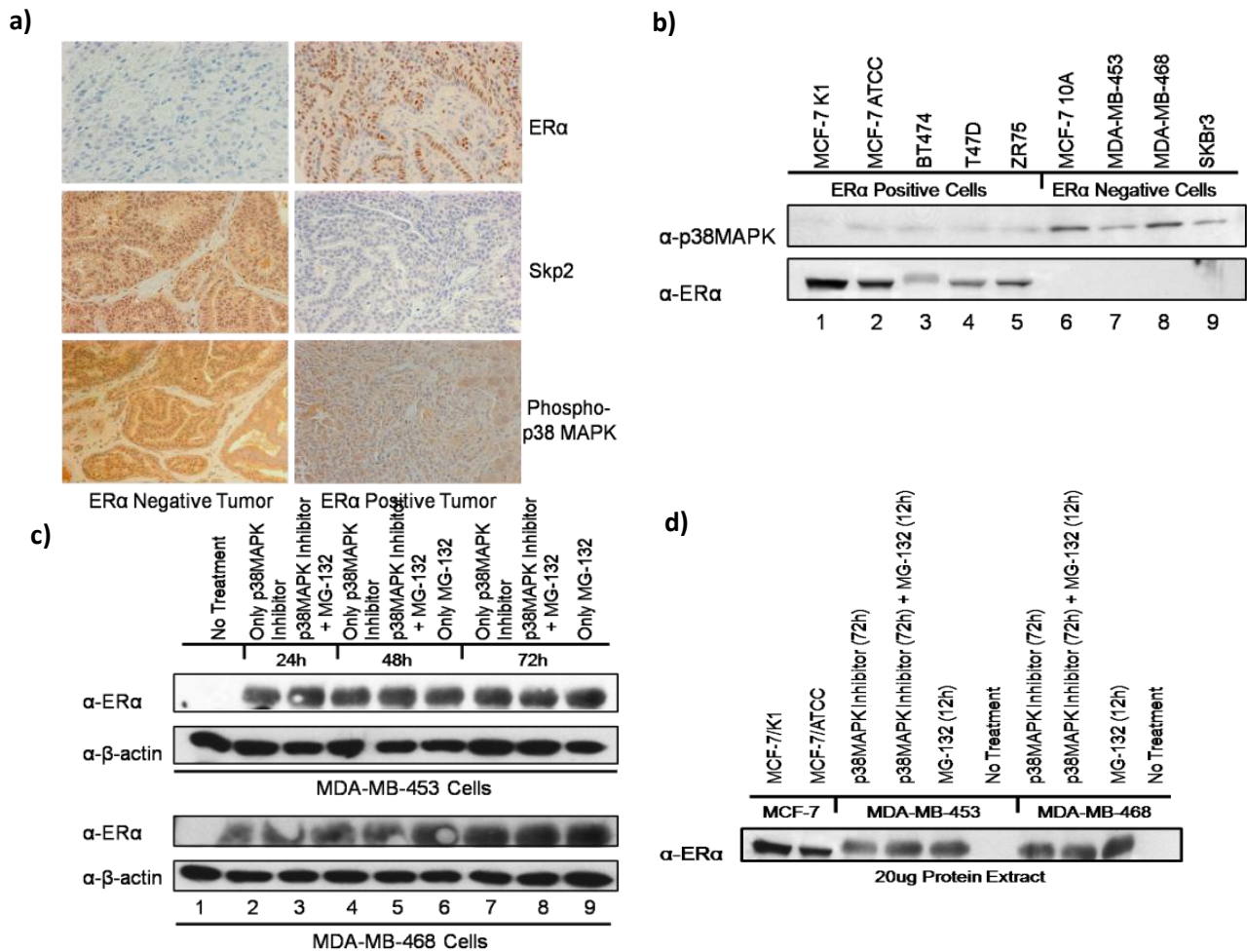
a) Cos-1 cells cultured in serum free media for two days and transfected with Skp2, ER $\alpha$ -WT or ER $\alpha$ -S294A mutant and HA-Ubiquitin expression vectors were pretreated with MG-132 (10 $\mu$ M) for 1h and thereafter treated with Veh (No Inhibitor), Cyclin Dependent Kinase 2 (CDK2) inhibitor (1 $\mu$ M) or p38MAPK inhibitor (1 $\mu$ M) for 4h in media containing serum before harvest, 24h post transfection. Cell lysates were immunoprecipitated using anti-ER $\alpha$  followed by SDS-PAGE and western blotting for ER $\alpha$ , Skp2 and HA. b) Endogenous Ubiquitination assay. MCF-7 cells were grown in 150-cm plates to 90% confluence. Adenovirus mediated over-expression of either Adeno- $\beta$ -Gal (Control) or Adeno-p38MAPK was performed followed 24h after by treatment with proteasome inhibitor MG-132 (10 $\mu$ M) for 5h followed by subsequent harvest in CoIP buffer (described previously in materials and methods). The cell lysate was subjected to immunoprecipitation using specific ER $\alpha$  antibody followed by SDS-PAGE and western blotting with anti-Ubiquitin (Santa Cruz) to detect ubiquitinated ER $\alpha$ , anti-ER $\alpha$  and anti-p38MAPK.



**Fig.3.8 p38MAPK Mediated Phosphorylation of ER $\alpha$  at Serine-294 is Required for Its Ubiquitination by Skp2**

a) Cos-1 cells cultured in serum free media for two days and transfected with Skp2, ER $\alpha$  and HA-Ubiquitin expression vectors along with increasing amounts of either p38MAPK-WT (wildtype) or p38MAPK-DN (dominant negative) were treated with MG-132 (10 $\mu$ M) for 5h before harvest, 24h post transfection. Cell lysates were immunoprecipitated using anti-ER $\alpha$  followed by SDS-PAGE and western blotting for ER $\alpha$ , Skp2, HA and p38MAPK. b) ZR-75 cells were infected with either control adenovirus (Adeno-Control) or Skp2 adenovirus (Adeno-Skp2) and 48h post infection treated with 1 $\mu$ M p38MAPK inhibitor for 6h and thereafter harvested. Cell lysates were prepared using RIPA buffer and SDS-PAGE followed by western blot analysis performed thereafter using anti-Skp2, anti-ER $\alpha$ , anti-p38MAPK and anti-phospho-p38MAPK antibodies. c) To perform in vitro kinase assay, 1 $\mu$ g of recombinant active phospho-GST-p38MAPK or inactive GST-p38MAPK protein (Invitrogen) was incubated with 1 $\mu$ l of in vitro translated ER $\alpha$ -WT or ER $\alpha$ -S294A protein as substrate, in the presence of 1X Kinase buffer from Cell Signaling Inc. supplemented with 200 $\mu$ M ATP in a total reaction volume of 50 $\mu$ l. The reactions were incubated at 30 $^{\circ}$ C for 30min for enzymatic catalysis to occur and thereafter terminated by addition of 3X SDS sample buffer and subjected to SDS-PAGE and western blotting using anti-phosphoserine, anti-ER $\alpha$ , anti-p38MAPK and anti-phospho-p38MAPK antibodies.

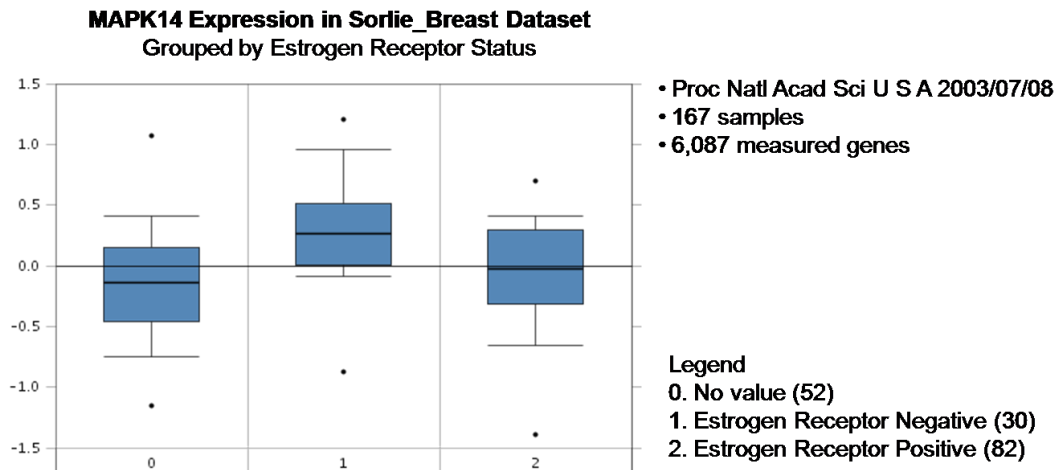




**Fig.3.9 p38MAPK Levels Inversely Correlate with ERα and Its Inhibition Restores ERα Protein In ER-negative Breast Cancer Cells**

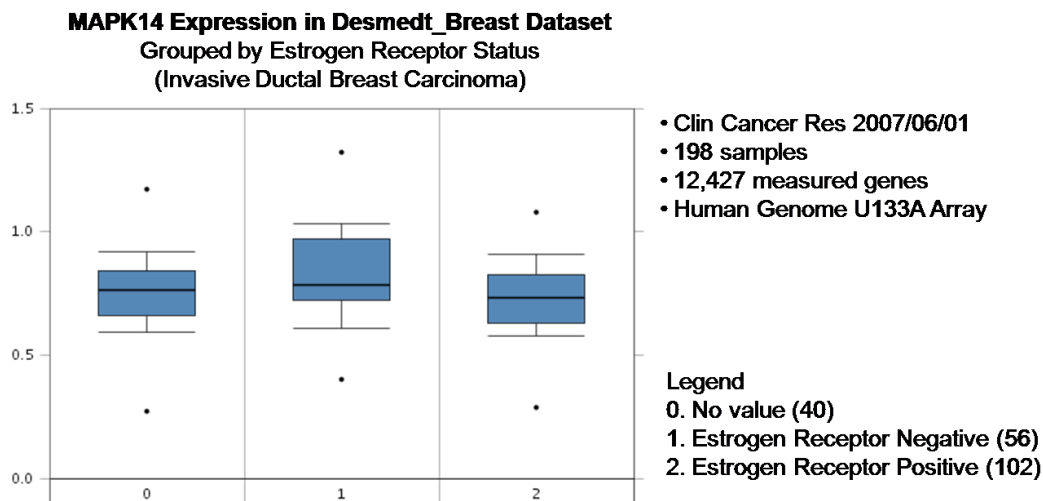
a) Paraffin embedded sections (0.2μM) from Breast Cancer patients with ER-negative and ER-positive tumors were stained for ERα, Skp2 and phospho-p38MAPK b) Relative expression levels of p38MAPK, Skp2 and ERα proteins were analyzed in breast cancer cell-lines by performing SDS-PAGE and western blot analysis. c) Two ERα negative cell-lines MDA-MB453 and MDA-MB468 were treated with vehicle or p38MAPK inhibitor (1μM) from Calbiochem for 24, 48 or 72h alone or in combination with 12h co-treatment with proteasome inhibitor MG-132 (10μM) or 12h treatment with proteasome inhibitor MG-132 (10μM) alone. Cells were thereafter harvested and subjected to lysis and protein extraction using RIPA buffer followed by SDS-PAGE analysis and subsequently western blotting with anti-ERα or anti-β-actin (loading control). d) Comparison of restored ERα protein in ER-negative cells to ERα in known amount of MCF-7 cells.

## SUPPLEMENTARY FIGURES



**S1(a). Oncomine Plots: p38MAPK or MAPK14 mRNA is Over-expressed in ER $\alpha$  Negative Human Breast Tumors.**

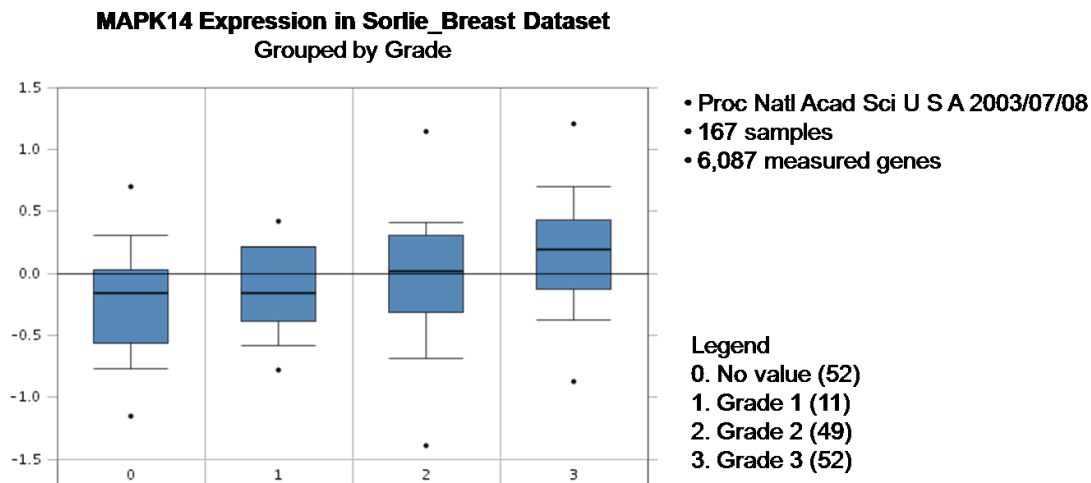
Box-plot from Sorlie\_Breast dataset of 167 human breast tumor samples, part of Oncomine database, grouped according to ER $\alpha$  status, showing higher expression of p38MAPK or MAPK14 gene in ER $\alpha$  negative tumors (30 samples) compared to ER $\alpha$  positive (82 samples), by microarray analysis (Human Genome U133A array). Note: \*P value < 0.05.



**S1(b). Oncomine Plot: p38MAPK or MAPK14 mRNA is Over-expressed in ER $\alpha$  Negative Human Breast Tumors.**

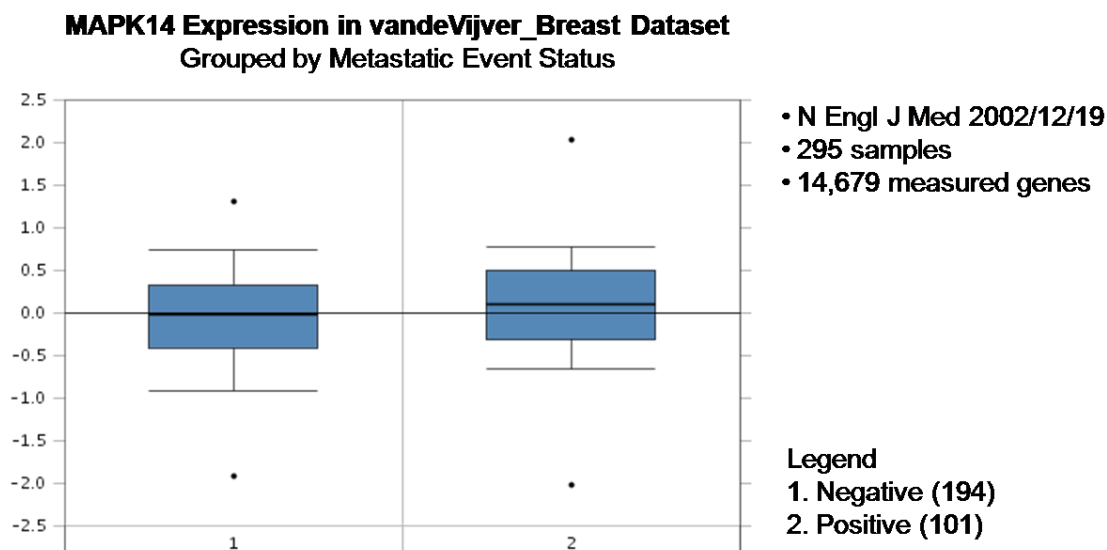
Box-plot from Desmedt\_Breast dataset of 198 human breast tumor samples, part of Oncomine database, grouped according to ER $\alpha$  status, showing higher expression of p38MAPK or MAPK14 gene in

ER $\alpha$  negative tumors (56 samples) compared to ER $\alpha$  positive (102 samples), by microarray analysis (Human Genome U133A array). Note: \*P value < 0.05.



**S2(a). Oncomine Plots: p38MAPK or MAPK14 mRNA is Over-expressed in Higher Grade Human Breast Tumors.**

Box-plot from Sorlie\_Breast dataset of 167 human breast tumor samples, part of Oncomine database, grouped according to tumor grade, showing higher expression of p38MAPK or MAPK14 gene in higher grade tumors (grade 2 and 3) compared to lower grade (grade 1), by microarray analysis (Human Genome U133A array). Note: \*P value < 0.05.



**S2(b). Oncomine Plot: p38MAPK or MAPK14 mRNA is Over-expressed in Highly Metastatic Human Breast Tumors.**

Box-plot from VandeVijver\_Breast dataset of 295 human breast tumor samples, part of Oncomine database, grouped according to occurrence or non-occurrence of a metastatic event, showing higher expression of p38MAPK or MAPK14 gene in metastatic tumors (101 samples) compared to non-metastatic (194 samples), by microarray analysis (Human Genome U133A array). Note: \*P value < 0.05.

## ACKNOWLEDGEMENTS

This work was supported by grants from the NIH RO1 CA18119 (BSK) and PO1 and The Breast Cancer Research Foundation (BSK).

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## **CHAPTER IV-EFFECT OF SKP2 AND ER $\alpha$ CROSS-TALK ON EESTRADIOL AND TAMOXIFEN DRIVEN GROWTH OF BREAST CANCER CELLS**

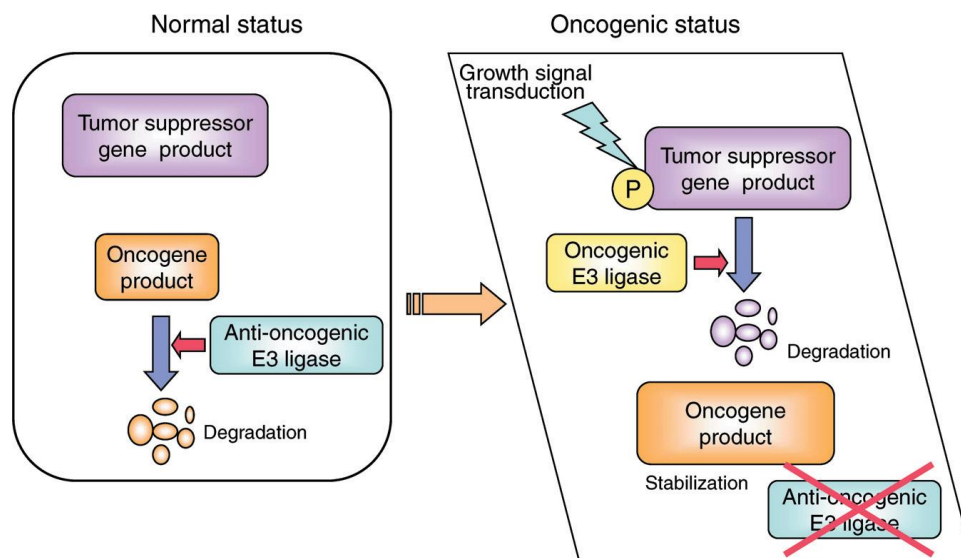
### **ABSTRACT**

Estrogen Receptor- $\alpha$  transcription complex consists of humongous population of factors with diverse biological functions and roles that extend vital contributions to the gene expression output. One of these factors identified by our work to modulate the expression of ER $\alpha$  target genes is Skp2 (S-phase Kinase associated protein-2), an E-3 ubiquitin ligase by function that was shown by our work to regulate the level of ER $\alpha$  in breast cancer cells via p38MAPK mediated phosphorylation dependent degradation. Surprisingly, Skp2 acted as a co-activator to ER $\alpha$  actions and was found to enhance the expression of some of the known ER-target genes, such as pS2, PgR, GREB1 by more than ~100 fold in the presence of either estradiol or tamoxifen. Interestingly, Skp2 was also able to significantly affect the basal gene expression of these ER $\alpha$  targets, one of which was the cdk-inhibitor and major regulator of the G1/S-checkpoint in the cell cycle, p21, known to be repressed by ER $\alpha$  upon estradiol or tamoxifen treatment. Further evidence of p21 regulation by Skp2 came from chromatin immuno-precipitation studies where we found recruitment of Skp2 on p21, but not p27, promoter along with ER $\alpha$ , similar to its recruitment on the promoters of other ER target genes such as pS2, PgR and GREB1.

Interestingly, p38MAPK mediated activation of Skp2 was required for this function as enhancement of ER $\alpha$  target gene expression by Skp2 was largely blocked in the presence of p38MAPK inhibitor or a dominant negative p38MAPK. Next we found by mass-spectrometry studies and in-cell kinase assays that p38MAPK phosphorylates Skp2 at Serine-64 position and the mutant of Skp2 that could not be phosphorylated at this position (Skp2-S64A) was incapable of regulating ER $\alpha$  target genes while the constitutively active Skp2-S64E mutant could. To this end we performed flowcytometry

analysis on estradiol treated MCF-7 cells that revealed enhanced entry of cells into the proliferative S-phase upon over-expression of either Skp2 or ER $\alpha$  and a much greater cooperativity upon co-expression. Our work therefore highlights Skp2 mediated ER $\alpha$  ubiquitination as an underlying mechanism for the activation of ER $\alpha$  mediated gene transcription to potentiate the mitogenic effects of estradiol in breast cancer cells.

## INTRODUCTION



Courtesy: Kitagawa K et al. Cancer Sci. 2009 Aug; 100(8):1374-81

**Fig.4.1 E3 Ubiquitin Ligases as tumor suppressors and oncoproteins**

Accumulation of oncogene products and enhanced degradation of tumor suppressor gene products in cancer cells. In normal cells, tumor suppressor gene products negatively regulate transformation and cancerous growth and oncogene products are quickly degraded by their E3 ligases, which target oncogenes as anti-oncogenic E3 ligases. Defects in the anti-oncogenic E3 ligase and overexpression of the oncogenic E3 ligases, which target tumor suppressor gene products, often promote carcinogenesis and cancerous growth.

### ***E3s as Oncogenes in Breast Cancer***

Many E3s could be oncogenes or tumor suppressor genes because frequent deregulation of E3s has been shown in human carcinogenesis. Some E3s have established roles in cell cycle and apoptosis, such as the Mdm2 and the SCFSkp2 complex. More recently discovered E3s, such as ARF-BP1, WWP1, and breast cancer–associated gene 2 (BRCA2), may be similarly important in breast tumorigenesis. These E3s are over-expressed in human breast cancer, and their inhibition leads to growth arrest or apoptosis [1].

#### **ARF-BP1/HUWE1**

The protein ARF-BP1 is a HECT domain E3 that targets the p53 protein for ubiquitin-mediated proteolysis. ARF-BP1 was shown to be a critical mediator of both the p53-independent and p53-dependent tumor suppressor functions of ARF. The gene encoding ARF-BP1 is located at Xp11, and gains on chromosomes Xp11-13 have been detected in breast cancer. In agreement with this, ARF-BP1 has been shown to be over-expressed in colorectal and breast cancer.

#### **WWP1**

WWP1 is located at 8q21, a region frequently amplified in human breast cancer. Indeed, copy number gain and over-expression is frequently detected in human prostate and breast cancer. WWP1 is also a HECT domain–containing E3. Growing evidence suggests that WWP1 negatively regulates the transforming growth factor- $\beta$  (TGF- $\beta$ ) tumor suppressor pathway by mediating the ubiquitination and degradation of Smad2, Smad4, and TGF- $\beta$  receptor 1. Besides inhibiting the above components of the TGF- $\beta$  pathway, WWP1 has recently been shown to function as an E3 for two transcription factors: KLF2 and KLF5. KLF2 and KLF5 have been shown to be frequently down regulated in ovarian and breast tumors, respectively. Both transcription factors were shown to induce apoptosis through regulating transcription of their target genes. WWP1 may also play a role in other biological processes, including regulation of epithelial sodium channels, viral budding, and receptor trafficking. Many studies have

found that WWP1 directly binds to specific targets through its WW domains, which can interact with a proline-rich motif in the target proteins. Alternatively, WWP1 targets Smad4 and TGF- $\beta$  receptor 1 through adaptor proteins Smad2 and Smad7, respectively.

### Mdm2

Gene amplification, mutation, over-expression, and aberrant splicing have been identified in 19 tumor types, including breast. It has been shown that expression of aberrantly spliced Mdm2 mRNA in breast carcinoma was associated with a shortened overall patient survival. Although Mdm2 has been characterized as a RING finger E3 for the tumor suppressor p53, Mdm2 also has transforming potential independent on the p53. Inhibiting the interaction of Mdm2 with p53 has therefore been a focus in drug discovery. Recently, several potent and selective small-molecule antagonists of Mdm2 have been identified.

### EFP

The EFP stimulates proliferation of breast cancer through facilitating ubiquitin-mediated destruction of a tumor suppressor 14-3-3 $\sigma$ . Recently, EFP was shown to function as an E3 ligase for 14-3-3 $\sigma$  in response to IFNs. EFP may regulate a switch from estrogen-dependent to estrogen independent growth of breast tumors because EFP over-expressed estrogen-dependent MCF-7 breast cancer cells gained the ability to form tumors in nude mice in the absence of estrogen. Inhibition of EFP expression by antisense oligonucleotides reduced tumor growth. The protein expression of EFP is significantly correlated with poor prognosis of breast cancer patients. Therefore, EFP may be a new valuable biomarker and molecular target for breast cancer.

### X-Linked Inhibitor of Apoptosis Protein/BIRC4

Several inhibitor of apoptosis proteins (IAP) possess RING finger domains that are able to bind E2s, ultimately promoting ubiquitination of target proteins. In addition to directly binding and inhibiting



caspases, IAPs may cause ubiquitination and subsequent proteolysis of caspases and other apoptotic regulators. For example, X-linked IAP (XIAP) targets caspase-3, caspase-7, and caspase-9 for degradation and may also enhance its own activity by mediating ubiquitination of its antagonists, including second mitochondria-derived activator of caspases (Smac). Over-expression of XIAP may cause the resistance to apoptosis induced by cytotoxic drugs in breast cancer. It has been shown that inhibition of XIAP by RNA interference suppresses MCF-7 xenograft tumor growth and sensitizes cells to etoposide and doxorubicin. Thus, the role of IAP proteins in ubiquitination is an emerging field with important implications for resistance to apoptosis in tumor cell chemotherapy.

#### *RING Finger Protein 11*

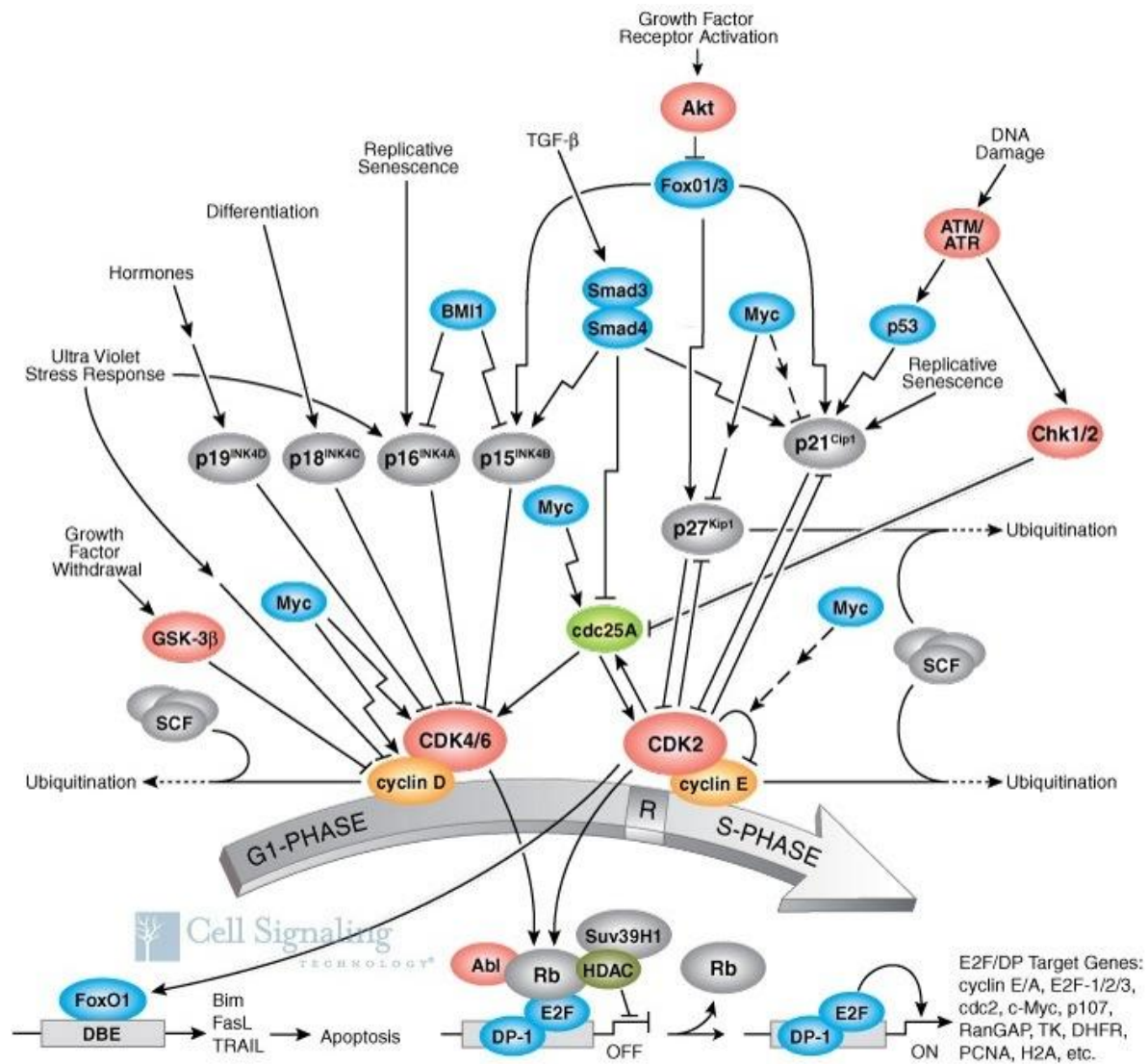
Both mRNA and protein of RING finger protein 11 (RNF11) are highly expressed in breast cancer cells, although its role in breast tumorigenesis remains to be elucidated. RNF11 was found to cooperate with Smurf2 to degrade an enzyme MSH, a positive regulator of both TGF- $\beta$  and epidermal growth factor receptor signal pathways. RNF11 interacts with multiple HECT-domain E3s and Cul1. Like the role of Skp1/2 in SCF E3, RNF11 may function as an adaptor to bridge some substrates to HECT domain E3s for ubiquitination because a large number of interacting partners have been revealed.

#### *BCA2/ZNF364*

BCA2 is another E3 with a RING domain. The BCA2 gene is located at 1q21.1, a region frequently amplified in breast cancer. Using immunohistochemistry and tissue microarray, Burger et al. showed that BCA2 protein was overexpressed in invasive breast cancer but correlated with positive estrogen receptor, negative lymph node metastasis, and increased survival. Inhibition of BCA2 by small interfering RNA suppressed T-47D and MCF7 cell proliferation. The targets of BCA2 E3 have not been identified.

## Skp2

The Skp2 gene is located at 5p13, a region found to be amplified in 11% of breast cancer cell lines in a comparative genomic hybridization study. Skp2 has been reported to be over-expressed in a subset of breast carcinomas (estrogen receptor and Her-2 negative), and Skp2 expression inversely correlates with p27 KIP1 levels in a variety of human tumors, including breast carcinomas [2]. Thus, Skp2 may be a potential specific biomarker and therapeutic target in a subset of aggressive breast carcinomas. Skp2 is an extensively studied F-box protein required for the ubiquitin-mediated degradation of the p27KIP1, p21CIP1, p130, and FoxO1 by SCF E3. Interestingly, the ubiquitination of p27KIP1 by SCFSkp2 requires an accessory protein Cks1. Recent structural studies show that Cks1 binds to the phosphorylated Thr187 side chain of p27KIP1. Expression of Cks1 is associated with decreased tumor differentiation and poor disease-free and overall survival outcome in breast cancer [3-6], [7-11].



Courtesy: [www.cellsignaling.com](http://www.cellsignaling.com)

#### Fig.4.2 Regulation of G1/S cell cycle checkpoint

The primary G1/S cell cycle checkpoint controls the commitment of eukaryotic cells to transition through the “gap” phase (G1) and enter into the DNA synthesis phase (S). Two cell cycle kinase-complexes, CDK4/6-cyclin D and CDK2-cyclin E, work in concert to relieve inhibition of a dynamic transcription complex that contains the retinoblastoma protein, Rb, and E2F.

### $\beta$ -TrcP1/BTRC1

$\beta$ -TrcP1 is another F-box protein but differs from Skp2 in that it harbors a  $\beta$ -transducin repeat domain. Over-expression of  $\beta$ -TrcP1 in mouse mammary gland epithelium promotes nuclear factor- $\kappa$ B activity and epithelial cell proliferation. Thirty-eight percent of transgenic mice develop tumors, including mammary, ovarian, and uterine carcinomas. Consistently, targeting of  $\beta$ -TrcP1 either by RNA interference or by forced expression of a dominant-negative  $\beta$ -TrcP1 mutant suppresses cell growth and sensitizes human breast cancer cells to the anti-proliferative effects of anticancer drugs.  $\beta$ -TrcP1 mediates ubiquitination and degradation of  $\beta$ -catenin, I $\kappa$ B, CDC25A, Smad4, and Emil1.

### Cul-4A

The Cul-4A gene is located at 13q34 and is frequently amplified and over-expressed in breast cancer. Recently, Cul4A protein was shown to participate in the Mdm2-mediated proteolysis of p53 and DET1-regulated c-Jun degradation. Additionally, Cul-4A expression is critical for early embryonic development because the homozygous deletion or heterozygous deletion of Cul-4A is lethal in a knock-out mouse model. In addition to the E3s described above, many other E3s have also been shown to be oncogenic proteins in breast cancer, such as E6-AP, COP1, and PirH2. All of these E3s target p53 for ubiquitin-mediated degradation.

### ***E3s as Tumor Suppressor Genes in Breast Cancer***

In contrast to oncogenic E3s, many other E3s, including BRCA1 and Fbw7, have been shown to be tumor suppressors in breast cancer. Frequently inactivating mutations or down-regulated expression of these E3s has been detected in breast cancer. Several recently discovered E3s, such as CHFR, SIAH1, and CHIP, may play a significant role in regulating breast tumorigenesis. Besides mutation and gene copy loss,

epigenetic alteration (i.e., promoter methylation) also contributes to inactivation of these tumor suppressors.

### BRCA1-BARD1

BRCA1 is a well-known tumor suppressor gene and is mutationally inactivated in familial forms of breast and ovarian cancer. BRCA1 protein contains a RING finger domain in its NH<sub>2</sub> terminus. This RING finger domain has been shown to auto-ubiquitinate and mono-ubiquitinate histone H2A in vitro. BRCA1 interacts with another RING finger protein, tumor suppressor BARD1, which is also mutated albeit with low frequency in breast cancer. BARD1 has BRCA1-independent and p53-dependent pro-apoptotic activity. BARD1 mRNA is lower in invasive breast cancers compared with normal breast tissues. Interestingly, BARD1 expression is highly up-regulated in the cytoplasm of most breast cancer cells and correlates with poor clinical outcome. The RING heterodimer BRCA1-BARD1 is a ubiquitin ligase, and the mutations in the RING finger domain of BRCA1 found in familial breast cancer abolish the E3 activity. The activity of BRCA1-BARD1 was shown to be down-regulated by CDK2 and enhanced by BRCC36 whose mRNA expression is elevated in breast tumors. BRCA1-BARD1 E3 was shown to ubiquitinate  $\gamma$ -tubulin, which regulates centrosome number and genome stability. Following that, the largest subunit of RNA polymerase II (Rpb1) was shown to be ubiquitinated by this E3 complex [12, 13].

### SIAH1

The SIAH1 gene was first identified as a candidate tumor suppressor gene at 16q12.1 in human hepatocellular carcinomas because of frequent loss of heterozygosity and expression down-regulation. Over-expression of SIAH1 in MCF7 cells suppresses cell growth by altering the mitotic process. SIAH1 is a RING finger E3 targeting  $\beta$ -catenin for ubiquitin-mediated degradation in response to activation of p53. The ubiquitin ligase complex contains SIAH1, SIAH1-interacting protein, Skp1, and the F-box protein Ebi. Importantly, this degradation pathway is different from degradation of  $\beta$ -catenin by SCF $\beta$ -TrcP. The

latter requires the  $\beta$ -catenin phosphorylation by glycogen synthase kinase 3 $\beta$ . It is well documented that  $\beta$ -catenin promotes cell cycle progression and inhibits cell cycle arrest. Because SIAH1 is a p53-induced gene, SIAH1-mediated  $\beta$ -catenin degradation may contribute to p53-dependent cell cycle arrest and apoptosis. In addition to  $\beta$ -catenin, SIAH1 has been reported to promote degradation of several substrate proteins, such as transcriptional coactivator OBF-1, transcriptional co-repressor CtIP, kinesin-like DNA-binding protein Kid, and cell fate regulator Numb.

### CHFR

Epigenetic inactivation of CHFR by aberrant promoter methylation was found in several types of carcinomas, including breast. In a knock-out mouse model, CHFR was suggested to be a tumor suppressor because mice deficient in its expression spontaneously develop a variety of epithelial tumors in major organs, including lung, liver, gastrointestinal tract, and reproductive system. The CHFR gene also encodes a RING finger domain containing E3, which was shown to play an important role in mitosis through targeting key mitotic proteins Plk and Aurora A for ubiquitin-mediated proteolysis.

### Parkin

Although mutations of the Parkin gene are the most common cause of hereditary parkinsonism, Parkin has also been suggested as a candidate tumor suppressor gene on chromosome 6q25-q27 in human breast cancer, ovarian cancer, and lung cancer. The expression of the Parkin gene has been shown to be frequently down-regulated or absent in breast tumor cells. Although no mutations or in vitro changes in cell proliferation or cell cycle were observed, forced expression of Parkin was shown to suppress tumorigenicity in nude mice. Parkin is a RING-type E3 involved in the ubiquitination pathway for misfolded proteins. Although  $\alpha$ Sp22, Pael receptor (Pael-R), and p38/JTV1 were identified as the Parkin substrates in brain cells, the targeted substrate in breast cancer cells remains unclear.

### Fbw7/hCDC4

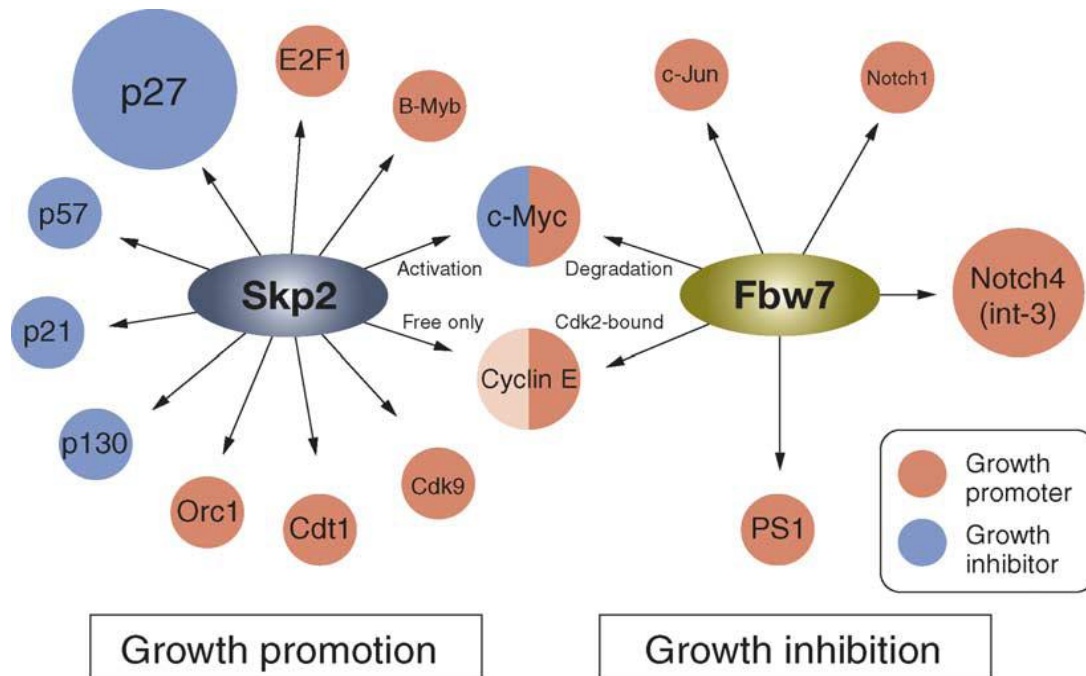
Human F-box protein Fbw7/hCdc4 has been reported to promote ubiquitin-mediated degradation of several oncoproteins involved in the control of cell division and growth, such as cyclin E, c-Myc, c-Jun, and Notch. Mice lacking Fbw7 die at about day 10 with multiple abnormalities. Although cyclin E expression is frequently dysregulated in breast cancer, a truncated mutant form of Fbw7, which can not bind to phosphorylated cyclin E target, has been found in one breast cancer cell line. However, the frequency of Fbw7 mutation in breast cancer has not been studied. Proteasomal degradation of cyclin E via the UPS is distinct from elastase, and calpain-mediated NH<sub>2</sub>-terminal proteolysis of cyclin E that generates low molecular weight forms of cyclin E, which are frequently detected in breast tumor tissue.

### CUL-5

CUL-5 is located at 11q22-23, which is frequently deleted in breast cancer. A statistically significant decrease in CUL-5 expression versus the matched normal tissue was detected in 82% (41 of 50) of the breast cancers. Over-expressing Cul-5 significantly attenuated cellular proliferation of the T-47D breast cancer cell line.

### CHIP/STUB1

The chaperone-dependent U-box E3 CHIP mediates a degradative pathway for ErbB2/Neu. ErbB2 over-expression is frequent in breast cancer and associated with poor prognosis. In addition to ErbB2, CHIP also promotes estrogen receptor  $\alpha$  and glucocorticoid receptor degradation and attenuates receptor-mediated gene transcription [14]. However, CHIP negatively controls the sensitivity of TGF- $\beta$  signaling by targeting Smads for ubiquitin-mediated degradation. Interestingly, CHIP was found to be associated with Parkin and enhances Parkin E3 activity. The CHIP gene is located at 16p13.3, which is frequently deleted in papillary carcinomas of the breast.



Courtesy: Keiichi I. Nakayama et al. Seminars in Cell & Dev. Biology Vol. 16, 3, 323-333, June 2005

**Fig.4.3 Various targets of Skp2 and Fbw7**

Size of circles represents proposed relative physiological importance of the substrates.

### ***Functional Mechanisms of E3s in Breast Cancer***

Every E3s pecifically targets one or several substrate proteins for ubiquitination. However, if an E3 plays an important role in breast tumorigenesis, the targets are usually involved in the cell cycle, p53 actions, transcription, DNA repair, signaling transduction, or apoptosis. All these cellular processes are known to be critical in breast cancer initiation and progression.

#### **Cell Cycle**

The cell cycle plays a central role in regulating cell proliferation and tumorigenesis. It is well documented that alterations of cell cycle regulators occur in breast cancer, such as over-expression of cyclin E and down-regulation of the p27KIP1. Because rapid protein turnover by ubiquitin mediated proteolysis is the



most important mechanism of controlling protein abundance for cell cycle regulators, the frequent genetic and expression alterations of their E3s contribute to breast tumorigenesis. Typically, cullin-based E3s, including SCF complexes, function through regulating the cell cycle. For example, F-box proteins Fbw7 and Skp2 target cyclin E and p27 KIP1 for degradation, respectively. In addition, APC/C facilitates proteolysis of cyclin B, securin, and several other proteins to regulate mitosis exit.

### p53

p53 is a key tumor suppressor targeted by multiple E3s, including Mdm2, ARF-BP1, E6-AP, Pirh2, p300, Topo1, and COP1. Interestingly, p53 itself induces expression of Mdm2 and COP1 to negatively control p53 activity. Besides Mdm2 and ARF-BP1, COP1, a RING finger-containing E3, is also significantly over-expressed in breast cancer. Additionally, Pirh2 protein expression is up-regulated in major human lung neoplasms. It would be interesting to know if Pirh2 is also over-expressed in human breast cancer.

### Transcription

Protein instability is a typical feature of many transcription factors, such as HIF1- $\alpha$ , Myc,  $\beta$ -catenin, c-Jun, and KLF5. Each of these transcription factors regulates expression of a large number of target genes, and alterations of these transcription factors are frequently involved in tumorigenesis. Besides proteolysis, accumulating evidence suggests that ubiquitination of transcription factors can enhance their transcriptional activation function. Therefore, it is not surprising that E3s of these transcription factors, such as von Hippel-Lindau, Fbw7, SIAH1, and WWP1, play important roles in tumorigenesis through controlling the abundance and activity of transcription factors [6, 15-17], [18-21].

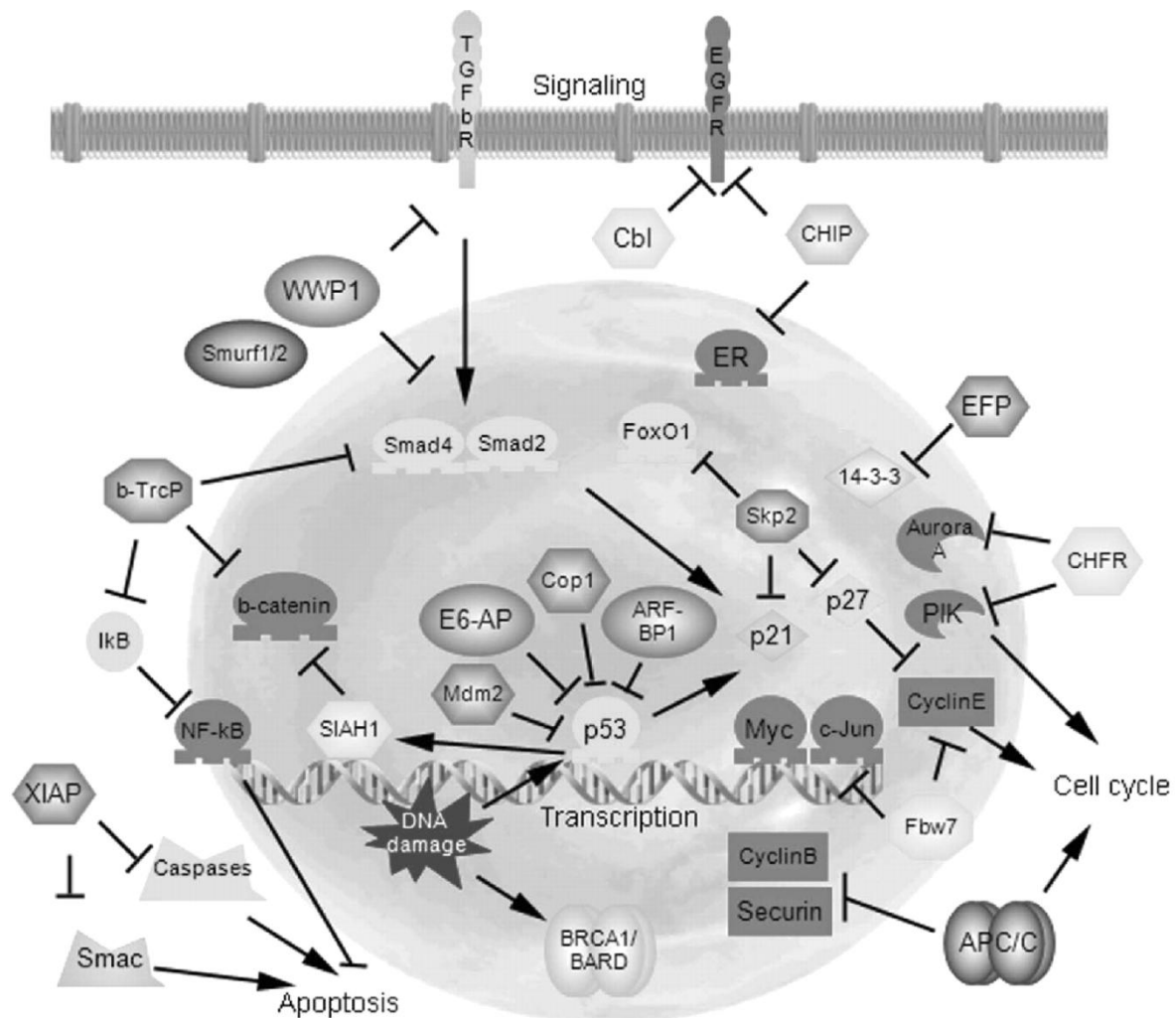
### DNA Repair

DNA damage is an important cause of gene mutation and carcinogenesis. Many proteins participating in DNA repair are regulated by the ubiquitin-proteasome pathway. For example, UV-induced p21 degradation is essential for DNA repair. Growing results indicate that BRCA1-BARD1 E3 is required for

double-strand break repair after exposure to ionizing radiation. Additionally, a BRCA1-dependent zinc finger transcriptional repressor ZBRK1 is degraded through the UPS upon DNA damage. Interestingly, altered expression of the ZBRK1 gene has been frequently found in human breast carcinomas. No E3 has been identified for ZBRK1 ubiquitination upon DNA damage, although RNF11 has been shown to be a ZBRK1-associated protein. Recently, DNA damage-induced degradation of Cdt1 and DDB2, two key proteins in DNA repair mechanisms, has been shown to link to Cul4A and DDB1. Therefore, compromise of DNA damage could be an important deregulating mechanism for BRCA1 and Cul4A in breast cancer.

### Growth Factor Signaling

Many growth factor signal receptors, such as epidermal growth factor receptor, estrogen receptor, and TGF- $\beta$  receptors, are frequently altered in breast cancer. For example, ErbB-2 is frequently amplified and over-expressed in breast tumors, and this alteration is associated with poor prognosis. In addition to CHIP, which promotes ligand-independent degradation of growth factor receptors, c-Cbl is another E3 that can negatively regulate the epidermal growth factor receptor by mono-ubiquitinating them for endocytosis upon ligand stimulation. It has been reported that degradation of ErbB-2 by Herceptin (a humanized ErbB-2 antibody) involves the recruitment of c-Cbl to ErbB-2. The Cbl gene is located at 11q23.3, a region frequently lost in breast cancer. Mutation of Cbl is oncogenic in many types of cancer. Additionally, controlling TGF- $\beta$  receptors by WWP1/Smurfs is implicated in the loss of sensitivity of tumorigenic cells to TGF- $\beta$ -induced growth inhibition.



Courtesy: Chen C. et al., Mol Cancer Res 2006;4(10). October 2006

**Fig.4.4 The well-studied E3s and their common mechanisms of regulating breast cancer**

↑, promoting; T, blocking. HECT-type E3s are in ellipse. RING finger E3s are in hexagon. F-box proteins are in octagon. Transcription factors are in O-vertex.

### Apoptosis

Inhibition and resistance to apoptosis is one of the major obstacles in cancer therapy. Apoptotic proteins have been identified as substrates of E3s and are frequently altered in breast cancer. In addition to p53 and the IAP family described above, IκB and Bcl2 family proteins are frequently deregulated by E3s in human cancers. It is well known that degradation of IκB by the SCFβ-TrcP complex is a critical step for

the activation of nuclear factor- $\kappa$ B and up-regulated expression of anti-apoptotic genes. Additionally, the pro-apoptotic proteins Bax, Bid, and Bim are degraded through the UPS, which is believed to be a survival mechanism in human cancer cells, although their E3 has not been revealed thus far. Up-regulated expression of Bim following detachment of normal breast epithelial cells from the extracellular matrix has been shown to be required for detachment-induced apoptosis. Recently, an anti-apoptotic Bcl-2 family member, Mcl-1, was shown to be an ARF-BP1 E3 substrate. Therefore, alteration of apoptosis by E3s is an important mechanism for breast carcinogenesis [22].

### ***Future Directions***

In conclusion, ubiquitin-mediated protein degradation plays an important role in many cancer-related cellular processes. E3s play critical roles because they control the substrate specificity. Accumulating evidence suggests that genetic and expression alteration of E3s contributes to breast carcinogenesis. In >500 human E3s, a number of E3s have been characterized as either oncogenes or tumor suppressor genes in breast cancer. Cell cycle, p53, transcription, signaling transduction, DNA repair, and apoptosis are major targets of E3s in breast cancer development. Several directions listed below should be important to develop E3s as diagnostic and therapeutic targets in the future.

### ***Identification of Novel Breast Cancer Related E3s as Biomarkers and Drug Targets-***

Although there are >500 E3s in the human genome, to date, only a limited number of E3s have been examined for their specific targets, genetic changes, and expression pattern in a large number of human breast tumors. High-throughput screening using E3 cDNA and small interfering RNA libraries have been developed to identify the cell cycle-related E3s. However, systematic screening of the E3s with genetic and expression alteration in human tumors has not been reported. With development of microarray technology, a cDNA chip with all E3s should be a useful tool to identify more cancer-related E3s. Tissue

microarray is another powerful tool to detect expression of a candidate E3 or its substrates simultaneously in large numbers of tumors by immuno-histochemical staining of a single microscope slide. An E3 with genetic and expression alteration in breast cancer could be developed as a biomarker for breast cancer diagnosis. Emerging technologies, such as array-based comparative genomic hybridization, cDNA microarray, tissue microarray, and RNA interference, will provide a better validation of many breast cancer-related E3s. It is anticipated that more breast cancer-related E3s will be identified in the future.

Human cancer	Correlation with poor prognosis	Correlation with low p27 levels	Refs
Biliary tract cancer	Yes	No	143
Breast cancer*	Yes	Yes	32, 42, 144–145
Cervical cancer	Yes	No	146
Colon cancer*	Yes	Yes	39, 43, 147–148
Endometrial cancer	Yes	ND	149–150
Gastric cancer*	Yes	Yes	45, 151–152
Glioma/glioblastoma <sup>‡</sup>	Yes	Yes	153–154
Kaposi sarcoma	Yes	No	155
Lung cancer (NSLC) * <sup>‡</sup>	Yes	Yes	156–158
Lung cancer (SLC)*	Yes	Yes	157–159
Lymphoma and leukaemia	Yes	No	40
Multiple myeloma	Yes	Yes	160–161
Melanoma	Yes	Yes	162–165
Oral cancers	Yes	Yes	147, 166–169
Ovarian cancer	Yes	Yes	170–171
Prostate cancer	Yes	Yes	34, 172–173

\*CKS1 (CDK subunit 1) also upregulated. <sup>‡</sup>Gene amplification. ND, not determined; NSLC, non-small-cell lung cancer; SKP2, S-phase kinase-associated protein 2; SLC, small-cell lung cancer.

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**Fig.4.5 Cancers associated with SKP2 deregulation**

\*CKS1 (CDK subunit 1) also upregulated. <sup>‡</sup>Gene amplification. ND, not determined; NSLC, non small-cell lung cancer; SKP2, S-phase kinase-associated protein 2; SLC, small-cell lung cancer.

### Identification of Specific Substrates for E3s-

Each E3 targets a small number of proteins for proteolysis. The target proteins for most E3s remain unclear. The identification of all substrates for all E3s is still a big challenge. Several strategies, including bioinformatics, in vitro expressing cloning, fusion protein, RNA interference, and arrays of synthetic phospho-peptides, certainly facilitate the identification of E3 substrates. A high-throughput screening to identify substrates for the yeast ubiquitin ligase Rsp5 has been reported recently. Because the E3s interact with their substrate proteins, maps of the interactome network generated from yeast, *Drosophila*, *Caenorhabditis elegans*, and human proteome by high-throughput yeast two-hybrid assays and newly developed mass spectrometric analysis technology will also facilitate the identification of the specific substrates for E3s.

### Development of Specific Inhibitors for E3s-

Approval of the general proteasome inhibitor Velcade by the Food and Drug Administration for the treatment of multiple myeloma suggests the promise of targeting the UPS in anticancer therapy. However, Velcade showed limited clinical activity against metastatic breast cancer and many side effects. Specific inhibitors of E3s should be highly specific drugs with few side effects because of the specificity of target recognition. The oncogenic E3s that target tumor suppressors could be potential targets for developing small molecule inhibitors. In contrast, the E3s with tumor suppressor function could also be promising targets for small-molecule activators. Several common approaches used for high-throughput screening for ubiquitin ligase inhibitors have been described recently. Moreover, high-throughput screening inhibitors for Mdm2-p53, Skp2-Cks1,  $\beta$ -Trcp1-I $\kappa$ B , and APC/C have been recently shown. Several companies are actively pursuing the inhibitors for E3s, such as Mdm2, Skp2, and Smurf. Furthermore, two inhibitors of Mdm2 actually were reported to have substantial p53-dependent anti-tumor effect in vivo. More detail of drug discovery in the UPS was discussed in a recent review. Although

E3s are believed to be ideal drug targets, targeting E3s is still in its infancy because no inhibitors have reached the clinic yet. The challenge remains to discover small molecular drugs that selectively inhibit the E3s. Emerging technologies, such as bioinformatics, high-throughput screening, structure-based drug design, and virtual library screening, will enhance the future E3-based drug discovery.

Reported substrates	Function	Upregulated in <i>Skp2</i> <sup>-/-</sup> MEFs	Ubiquitylation reconstituted <i>in vitro</i>	Refs
p27*	Cell cycle control	Yes	Yes	12, 13, 14
p21*	Cell cycle control	Yes	Yes	21, 22, 126
p57	Cell cycle control	Yes	Yes	23
Cyclin A	Cell cycle control	Yes	No	18
Cyclin E <sup>‡</sup>	Cell cycle control	Yes	Yes	18
Cyclin D1	Cell cycle control	Yes	No	22
CDT1	DNA replication	No	Yes	127
ORC1	DNA replication	ND	No	128
BRCA2	DNA repair	ND	No	129
RAG2	DNA repair	ND	Yes	130
TOB1	Gene transcription	Yes	Yes	24
RBL2*	Gene transcription	ND	Yes	26
FOXO1	Gene transcription	ND	Yes	27
MEF/ETS	Gene transcription	ND	Yes	131
MLL	Gene transcription	ND	Yes	132
MYB	Gene transcription	ND	No	133
MYC <sup>§</sup>	Gene transcription	ND	No	134–135
E2F1	Gene transcription	No	No	136
HPV-E7	Viral oncogenesis	Yes	Yes	137
USP18	Interferon signaling	Yes	No	28
MKP1	ERK signalling	ND	Yes	138
SMAD4	Signal transduction	ND	Yes	139
CDK9	Transcriptional elongation	Yes	No	29–30
E2A	B- and T-cell development	ND	No	140–141
TAL1	Erythroid differentiation	ND	No	142
RASSF1	Microtubule stabilizer	Yes	Yes	25

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**Fig.4.6 Reported substrates of SKP2**

\*Requires CKS1 (CDK subunit 1) for binding to SKP2. ‡Only free (CDK-unbound) cyclin E. §Ubiquitylation seems to promote transcriptional activity. BRCA2, breast cancer associated 2; CDK9, cyclin-dependent kinase 9; CDT1, chromatin licensing and DNA replication factor 1; FOXO1, Forkhead box-containing, O

subfamily 1; HPV-E7, human papillomavirus E7 protein; MEFs, mouse embryonic fibroblasts; MEF/ETS, myeloid ELF1-like factor; MKP1, mitogenactivated protein kinase (MAPK) phosphatase 1; MLL, myeloid/lymphoid leukaemia; ND, not determined; ORC1, origin recognition complex 1; RAG2, recombination activating gene 2; RASSF1, Ras association domain family 1; RBL2, retinoblastoma-like 2 (also known as p130); SKP2, S-phase kinase-associated 2; TAL1, T-cell acute lymphocytic leukaemia 1 (also known as SCL); TOB1, transducer of ERBB2; USP18, ubiquitin-specific peptidase 18 (also known as UBP43).

## **EXPERIMENTAL PROCEDURES**

### ***Antibodies-***

Anti-Skp2 (N-19, H-435, A-2), anti-ER $\alpha$  (HC-20, F-10), anti-p21 and anti-p27 were purchased from Santa Cruz Biotechnology. Anti-p38MAPK (9212, 9228) and anti-phospho (Thr180/Tyr182) p38MAPK (9211, 9216) were from Cell Signaling, anti-flag rabbit and mouse from Sigma and anti-phosphoserine (ab17465) were purchased from Abcam. Horseradish peroxidase (HRP)-conjugated donkey anti-goat, donkey anti-mouse and donkey anti-rabbit IgG secondary antibodies were from Santa Cruz Biotechnology.

### ***Cell Culture and Transfections-***

MCF-7 cells were maintained in growth medium containing Minimum Essential Medium (MEM)-with phenol red supplemented with 5% heat inactivated Calf Serum (CS), 100 units per ml of Penicillin and 50  $\mu$ g/ml of Streptomycin at 37°C in non-CO<sub>2</sub> incubator or treatment medium containing Minimum Essential Medium (MEM)-without phenol red supplemented with 5% Charcoal dextran stripped Calf Serum (CS) at 37°C under 5%CO<sub>2</sub> in humidified air. MCF-7-tamr cells (tamoxifen resistant derivatives of MCF-7 cells) were maintained in similar conditions as described for MCF-7 cells except they were grown in the presence of 1 $\mu$ M TOT included in growth media formulation. ZR-75 cells were maintained in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 100 units per ml of Penicillin and



50 µg/ml of Streptomycin at 37°C and 5%CO<sub>2</sub> in humidified air. All transient plasmid transfections were performed using LipofectAMINE™ 2000 Reagent from Invitrogen according to manufacturer's instructions. siSkp2 transfection was performed using Lipitoid reagent kindly provided by Dr. Ronald Zuckermann at the Molecular Foundry, Lawrence Berkeley National Laboratory (23). All other siRNA transfections were performed using Dharmafect transfection reagent from Dharmacon.

### ***Plasmids-***

Skp2 cDNA was purchased by Open Biosystems and cloned into pcDNA3-Flag expression vector. Various Skp2 point mutants, Skp2-S64A and Skp2-S64E, were generated from this full-length construct by site-directed mutagenesis strategy (kit purchased from Stratagene) and PCR based subcloning. ERα was cloned into either pcDNA3-Flag expression vector or pcDNA3.1-Myc/His expression vector from Invitrogen. ERα point mutant, ERα-S294A was generated from the full-length Flag-tagged construct by site-directed mutagenesis strategy (kit purchased from Stratagene) and PCR based subcloning, similar to that done for Skp2 (as described earlier). pMEV-2HA-p38MAPK-WT (wildtype) and DN (dominant negative) mutant were purchased from Biomyx Technology. Adenoviral vectors encoding Skp2-WT or ΔF-box mutant, p38MAPK-WT (wildtype) or DN (dominant negative) mutant and ERα-WT were constructed by cloning relevant sequences into pAdTrack vector purchased from Stratagene. Details for generation of any of the above mentioned expression vector, is available upon request.

### ***Silencing by Small Interfering RNA (siRNA)-***

MCF-7 or ZR-75 cells seeded onto 6-well plates at a confluence of 40-60% were transfected with siRNA duplexes, siSkp2 (Qiagen) or si-p38MAPK (Dharmacon), or non-targeting control siGL-3 (Dharmacon or Qiagen). siSkp2 transfection was performed using Lipitoid reagent kindly provided by Dr. Ronald Zuckermann at the Molecular Foundry, Lawrence Berkeley National Laboratory (23, 24). All other siRNA transfections were performed using Dharmafect transfection reagent from Dharmacon. Adenovirus

Construction and Infection for Over-expression of Endogenous Factors-cDNAs for the genes of interest, namely Skp2-WT, Skp2 $\Delta$ Fbox, p38MAPK-WT, p38MAPK-DN (dominant negative) and ER $\alpha$  were first cloned into pAdTrack-CMV shuttle vector from Stratagene using PCR based subcloning. DNA from positive clones was linearized by digestion with PmeI and electroporated into electrocompetent BJ5183 cells (Stratagene) already containing plasmidP1 for the recombination event to occur. Recombined DNA was thereafter extracted and transfected into AD293 cells for adenovirus preparation. After rounds of amplification the virus obtained was then purified by CsCl density gradient centrifugation and characterized for its titre and ability to over-express the protein of interest. MCF-7 cells seeded onto 6-well plates at a confluence of 40-60% were thereafter infected with this virus or an equal MOI (Multiplicity of Infection) of a non target control virus followed by ligand treatment (if required). Cells were harvested and processed for RNA and/or protein extraction as per requirement.

#### ***Gene expression analysis by Quantitative Real-time PCR-***

MCF-7 cells seeded onto 6-well plates at a confluence of 40-60% were either siRNA transfected to silence the gene of interest or infected with adenovirus to over-express the gene of interest followed by treatment with ligand if necessary. RNA was isolated using Trizol reagent (Invitrogen) following manufacturer's protocol. The mRNA for genes of interest was quantitated by SYBr Green-based reverse transcriptase-PCR (RT-PCR) using the ABI7600ht Sequence Detection System (Applied Biosciences). All mRNA quantities were normalized against 36B4 and primer sequences are available upon request.

#### ***Synchronization of MCF-7 cells-***

MCF-7 cells were plated in MEM supplemented with 5% Calf serum. To synchronize in the G1 phase, the cells were starved for 48h, fed with 10% serum containing medium, and then treated for 24h with 100 $\mu$ M Indole 3-carbinol. Indole 3-carbinol, a chemical that naturally occurs in Brassica vegetables, induces a G1 cell cycle arrest by inhibiting Cdk6 expression (Narayanan et al. 2005). To synchronize in

the S-phase, cells were serum starved for 48h to first synchronize in the G0/G1 phase and then synchronized at the G1/S boundary by incubation with 1.5µg of 4-Hydroxyurea/ml media for 24h in 10% serum containing medium. 4-Hydroxyurea, a specific inhibitor of DNA synthesis, blocks cell cycle at the G1/S boundary by inhibiting the enzyme Ribonucleotide Reductase. The cells were thereafter washed with Hank's buffered saline (HBS) and released into S-phase with serum free medium. Synchronization in the G2/M phase was carried out by synchronizing the cells at the G1/S boundary with 1.5µg of 4-Hydroxyurea/ml media for 48h in 10% serum containing medium, washing with HBS, and synchronizing in the G2/M phase by treating with 1µg/ml Nocodazole in 10% serum containing medium for 24h. Nocodazole is an anti-microtubule agent that arrests cells in the G2/M phase of the cells cycle by inhibiting microtubule polymerization and therefore mitotic spindle formation.

#### ***FACS analysis of MCF-7 cells-***

MCF-7 cells were washed once in cold Phosphate buffered saline (PBS), scraped in PBS and pelleted by centrifugation at 340g in a Beckman centrifuge at 4°C. The cells were resuspended in 2ml of 0.9% Sodium chloride and fixed for 30min in 5ml of 90% Ethanol. Ethanol was added dropwise with constant vortexing. After 30min, the cells were centrifuged out of the fixing solution and resuspended in 1ml Propidium Iodide (50µg/ml) diluted in PBS and supplemented with 100µg of RNase A (Roche). The cells were incubated at 37°C for 15min and cell cycle distribution was then analyzed by flow cytometry. FACS analysis was performed for each treatment variable to verify the synchronization of cells in different phases of the cell cycle.

#### ***Luciferase Reporter Gene Assay-***

MCF-7 or Hec-1 cells were transfected with plasmids of interest using LipofectAMINE 2000 following manufacturer's protocol. The dual luciferase assay system used was purchased from Promega Corp. (Madison, WI). Promoters for various ER target genes were cloned into 2(ERE)-pS2-Luciferase gene-

pCMV plasmid upstream of the pS2 gene and transfected along with Renilla luciferase gene plasmid (Clontech, Palo Alto, CA) and various combinations of ER $\alpha$  and/or Skp2-WT, Skp2-S64A, Skp2-S64E. Luciferase values were normalized using renilla luciferase gene as internal control for transfection efficiency. The amounts of plasmids transfected were: 1 $\mu$ g of the promoter constructs, 50 ng of Renilla luciferase construct, and 100 ng of ER $\alpha$  construct. Every experiment was performed at least three independent times.

### ***Chromatin Immunoprecipitation (ChIP) and sequential ChIP (reChIP) assays-***

Chromatin Immunoprecipitation assays (ChIPs) were performed with minor modifications as described in Metivier et al. (Gannon 2003). MCF-7 cells were weaned in minimum essential medium (MEM) supplemented with 5% Charcoal Dextran (CD)-stripped calf serum for 4 days followed by treatment with vehicle (ethanol), E2 (10nM) or Tamoxifen (1 $\mu$ M) for 45', 4h or 24h. In some experiments this ligand treatment was preceded by 48h long infection with Adeno-Skp2-WT or Adeno-Skp2 $\Delta$ Fbox, Adeno-p38MAPK-WT or Adeno-p38MAPK-DN, or equal MOI of a control adenovirus. Antibodies used for pull-downs were purchased from Santa Cruz Biotechnology [ER $\alpha$  (HC-20), Skp2 (N-19), RNA polymerase II (N-20), Abcam [RNA polymerase II (ab817), RNA polymerase II phospho-Ser2 (ab5095), RNA polymerase II phospho-Ser5 (ab5131)] and Bethyl Laboratories Inc. [p38MAPK (A310-212A)]. The DNA isolated was subjected to quantitative real-time PCR using gene specific primers (sequences available upon request) while using 36B4 as internal control (and measuring total input DNA in every sample) and a recruitment index was calculated (ratio between specific antibody signal over IgG signal). ChIP/reChIP experiments were done following the same ChIP protocol. After the first pull-down, immunoprecipitated material was recovered with 10mM dithiothreitol in IP buffer at 37°C for 30min, diluted and subjected to a second round of immunoprecipitation.

### ***Cell Proliferation assay-***

WST-1 cell proliferation assay is a colorimetric assay based on the cleavage of a tetrazolium salt, MTS, by mitochondrial dehydrogenases to form formazan in viable cells following the addition of WST-1, which can be detected by measuring absorbance at 450nm, which in turn is used as a measure of cell proliferation and viability. MCF-7-tamr cells were counted using a hemocytometer and seeded at 50,000 cells/well in a 12-well plate. Seeded cells were incubated overnight at 37°C with 5% CO<sub>2</sub> in a humidified atmosphere and next day treated with ligand, vehicle (ethanol), E2 (1nM) or Tamoxifen (1μM) and harvested after 0, 2, 4 and 6 days of treatment. At the time of harvest media was aspirated and 500μl of WST-1 reagent diluted 1:10 with 1XMEM supplemented with 5% CD stripped-Calf serum was added to each well. Incubation was continued for an additional 30min at 37°C with 5% CO<sub>2</sub> in a humidified atmosphere before absorbance was measured at 450nm versus a 650nm reference to compare the experimental samples to control, in Spectra MAX 190 microplate reader with SoftMax Pro software from Molecular Devices. Results were analyzed by exporting the data to MS Excel and expressing the mean ± SD for each sample, while every sample was run in triplicate.

## **RESULTS AND EXPERIMENTAL FINDINGS**

### ***Skp2 Positively Regulates ERα Target gene Expression***

Members of the ubiquitin-proteasome machinery have been implicated in regulating transcriptional functions of nuclear receptors and their co-regulators. Since Skp2 and ERα are both known to enhance the proliferation of MCF-7 breast cancer cells in the presence of estradiol, we tested the possibility of a role for Skp2 in mediating ubiquitination dependent regulation of ERα function, thereby driving progression of these cells into S-phase. For this we performed adenovirus mediated over-expression of Skp2, p38MAPK or ERα in MCF-7 cells followed by treatment with ligand, estradiol or

tamoxifen. Gene expression profiling of various ER $\alpha$  target genes known to play important roles in regulating growth and proliferation of these cells (pS2, PgR, GREB1) showed highly elevated basal expression with further enhancement in the presence of estradiol or tamoxifen, upon over-expression of either Skp2 or p38MAPK. Skp2, which is known to down-regulate p21 translationally, was interestingly found to enhance ER $\alpha$  mediated transcriptional down-regulation of the gene as well both in the absence and presence of ligand, estradiol or tamoxifen (Figure 4.7a) and this also reflected on the protein level (Figure 4.7b). This mRNA reduction (p21) and increase (pS2, GREB1, PgR) was reversed in the presence of either ICI (Figure 4.8a) or a specific siRNA directed against ER $\alpha$  (Figure 4.9a, b and c), thereby suggesting the phenomenon to be ER $\alpha$  dependent. Of note, this transcriptional regulation by Skp2 was specific for p21 and was not detected for p27, another closely related cyclin dependent kinase inhibitor, also a translational target of Skp2. Also, Skp2-mutant (Skp2 $\Delta$ Fbox) unable to degrade ER $\alpha$  or p38MAPK-mutant (p38MAPK-DN) unable to phosphorylate it blocked the Skp2 or p38MAPK mediated enhancement of transcriptional down-regulation of p21 gene by estradiol bound ER $\alpha$  (Figure 4.8b).

### ***Skp2 Regulates ER $\alpha$ Target genes by Getting Recruited to Their Promoters***

We next wanted to check if this transcriptional regulation of ER $\alpha$  target genes by Skp2 and p38MAPK is due to their recruitment to the gene promoters. For this we performed Chromatin Immunoprecipitation (ChIP) assay and found recruitment of both Skp2 and p38MAPK to ER $\alpha$  target gene promoters regulated at the mRNA level (pS2, PgR, GREB1, p21) but not on those that were not (p27) (Figures 4.10a, b and 4.11a, b). By ChIP re-ChIP we were able to show the presence of Skp2 and p38MAPK together with ER $\alpha$  transcriptional complex on target gene promoter (Figure 4.12a and b).

### ***Skp2 Potentiates the Effects of Estradiol Bound ER $\alpha$ In Driving S-phase Entry of MCF-7 Cells***

Interestingly, flow-cytometry analysis to check the effect of over-expression of Skp2 alone or Skp2 and ER $\alpha$ -WT on the capacity of MCF-7 breast cancer cells to enter S-phase in the presence of

estradiol showed cooperativity between ER $\alpha$  and Skp2-WT in promoting progression of cells through G1/S checkpoint and their entry into S-phase while the Skp2-mutant (Skp2 $\Delta$ Fbox) that is unable to degrade ER $\alpha$  blocked this cell cycle progression (Figure 4.13a and b). This Skp2 driven enhancement of mitogenic effects of estradiol were through ER $\alpha$  as they were blocked in ER $\alpha$  negative MDA-MB-468 cells (Figure 4.14a and b). These findings that show Skp2 mediated ER $\alpha$  ubiquitination/ turnover to increase the magnitude of ER $\alpha$  target gene regulation thereby facilitating the entry of cells into S-phase could underlie the rather poorly understood phenomenon behind more aggressive ER-negative tumors compared to ER-positive, as increased ER $\alpha$  turnover by Skp2 and hence its lower level through G1/S-checkpoint seems to facilitate the progression of cells into S-phase.

### ***Skp2 Interacts With and Ubiquitinates ER $\alpha$ Specifically in the G1/S and S-phases of the Cell Cycle***

Since Skp2 is a key regulator of cell cycle progression and regulates entry of cells into S-phase through a specific yet coordinated control of factors that regulate the G1/S checkpoint, we wanted to investigate if its interaction with and regulation of ER $\alpha$  is cell cycle stage dependent. We therefore synchronized MCF-7 cells in early G1, G1/S, S and G2/M phases of the cell cycle using the indicated scheme (Figure 4.15a) and monitored interaction between Skp2 and ER $\alpha$  along with the levels of the two proteins and also of p38MAPK which our data showed to be regulating Skp2 mediated ER $\alpha$  degradation. FACS analysis was performed in a parallel experiment for each treatment variable to verify the synchronization of cells in different phases of the cell cycle.

The cell synchronization in the early G1 phase ranged between 85-97% for G1 synchronized cells compared to about 66% in asynchronous cells, across multiple experiments. Synchronization in the G1/S and S-phase boundary ranged between 40-50% for G1 synchronized cells and about the same for S-phase arrested cells compared to about 66% and 16% respectively in asynchronous cells.

Synchronization in the S-phase ranged between 55-65% for S-phase cells compared to about 16% in asynchronous population and that in G2/M phase was between 80-99% compared to 17% in asynchronous cells (Figure 4.15b). We found that Skp2 levels were low in G1, in accordance with previous reports, and that Skp2 failed to interact with ER $\alpha$  at this stage in cell cycle. Interaction of ER $\alpha$  with Skp2 could be detected starting from the G1/S phase, peaking at S-phase and thereafter declining as cells progressed into the G2/M phase (Figure 4.16b). Interestingly, we found ER $\alpha$  turnover by Skp2 occurs specifically in the G1/S and S-phases of the cell cycle and this was also when the activation of p38MAPK was seen to be coincidentally up (Figure 4.16a). As there have been previous reports for these E3-ubiquitin ligases to regulate nuclear receptor mediated trans-activation of target genes we wanted to check the effect of over-expression of Skp2 on transcription regulation by ER $\alpha$ . We synchronized MCF-7 cells in the various stages of the cell cycle (as described earlier) and performed reporter gene analysis by transfecting the (ERE)<sub>2</sub>-pS2-Luciferase reporter plasmid in the presence of ER $\alpha$  with or without over-expressed Skp2 in the presence of vehicle or estradiol and found a significant increase in ER $\alpha$  mediated target gene expression in the G1/S and S-phases of the cell cycle which seemed to be Skp2 and estradiol dependent (Figure 4.16c). The data suggests that the regulation of ER $\alpha$  levels by Skp2 which occurs largely in the G1/S and S-phases of the cells cycle to be important for the trans-activation functions of the nuclear hormone receptor.

## DISCUSSION

Having established a novel ER $\alpha$  phospho-degron code involving its phosphorylation by p38MAPK at Serine-294 and implicating it in the turnover of the receptor by Skp2, we investigated the biological significance of this regulation in breast cancer. Since both Skp2 and ER $\alpha$  have been reported to have critical roles in regulating cell proliferation and oncogenic transformation (Weiderpass E et al., 2000;



Hayashi S.I et al., 2003; Tanaka Y et al., 2003; Yager J.D et al., 2006; Girard G.M et al., 2007; Chae Y.K et al., 2009; Latres E et al., 2001; Signoretti S et al., 2002; Shapira M et al., 2004; Moro L et al., 2006; Sanada T et al., 2004; Traub F et al., 2006; Sonoda H et al., 2006; Gstaiger M et al., 2001; Kamata Y et al., 2005; Lahav-Baratz S et al., 2004; Ma X.M et al., 2006; Yokoi S et al., 2004; Zhu C.O et al., 2004) and estrogens are known to elicit mitogenic effects in the breast through ER $\alpha$ , we show the role of Skp2 mediated ER $\alpha$  turnover in regulating cell cycle progression in these cells. Intriguingly, Skp2 and ER $\alpha$  cooperate in driving the progression of these cells into the proliferative S-phase, a phenomenon reversed by the dominant negative Skp2 $\Delta$ Fbox (incapable of degrading ER $\alpha$ ), supporting the hypothesis for Skp2 mediated ER $\alpha$  turnover in the G1/S-checkpoint phase, driving the entry of these cells into the S-phase.

Consistent with our observations, many recent studies have shown the requirement of continuous exchange and turnover of the nuclear receptors and co-regulatory proteins on the target gene promoter for a successful transcription cycle to occur (Reid G et al., 2002, 2003; Metivier R et al., 2003; Lipford and Deshaies, 2003; Muratani and Tansey, 2003; Baker and Grant, 2005; Dennis and O'Malley, 2005). Chromatin immunoprecipitation (ChIP) assays have shown that liganded ER $\alpha$  mediated formation of transcription complexes on gene promoters is ordered and involves sequential association and dissociation of ER $\alpha$  (Shang et al., 2000; Burakov et al., 2002). Moreover, inhibiting proteasome activity impairs ER $\alpha$  mediated transcription and immobilizes it to the nuclear matrix (Stenoien et al., 2001). These insights together with our observation that the ER $\alpha$ /Skp2 interaction occurs mainly in the cell nucleus prompted us to study the influence of Skp2 on ER $\alpha$  induced transcription. Since mitogenic effects of estrogens on growth are mediated largely through impact on ER $\alpha$  target gene expression, investigating this may offer a unique role for Skp2 mediated ER $\alpha$  turnover as an underlying molecular mechanism to potentiate cell cycle progression. Interestingly, we observed that Skp2 enhanced the ability of ER $\alpha$  to induce transcription of its target genes whereas Skp2 mutant deficient in E3 ligase

function failed to do so thereby supporting our hypothesis of a tight correlation between degradation of ER $\alpha$  and its trans-activation function.

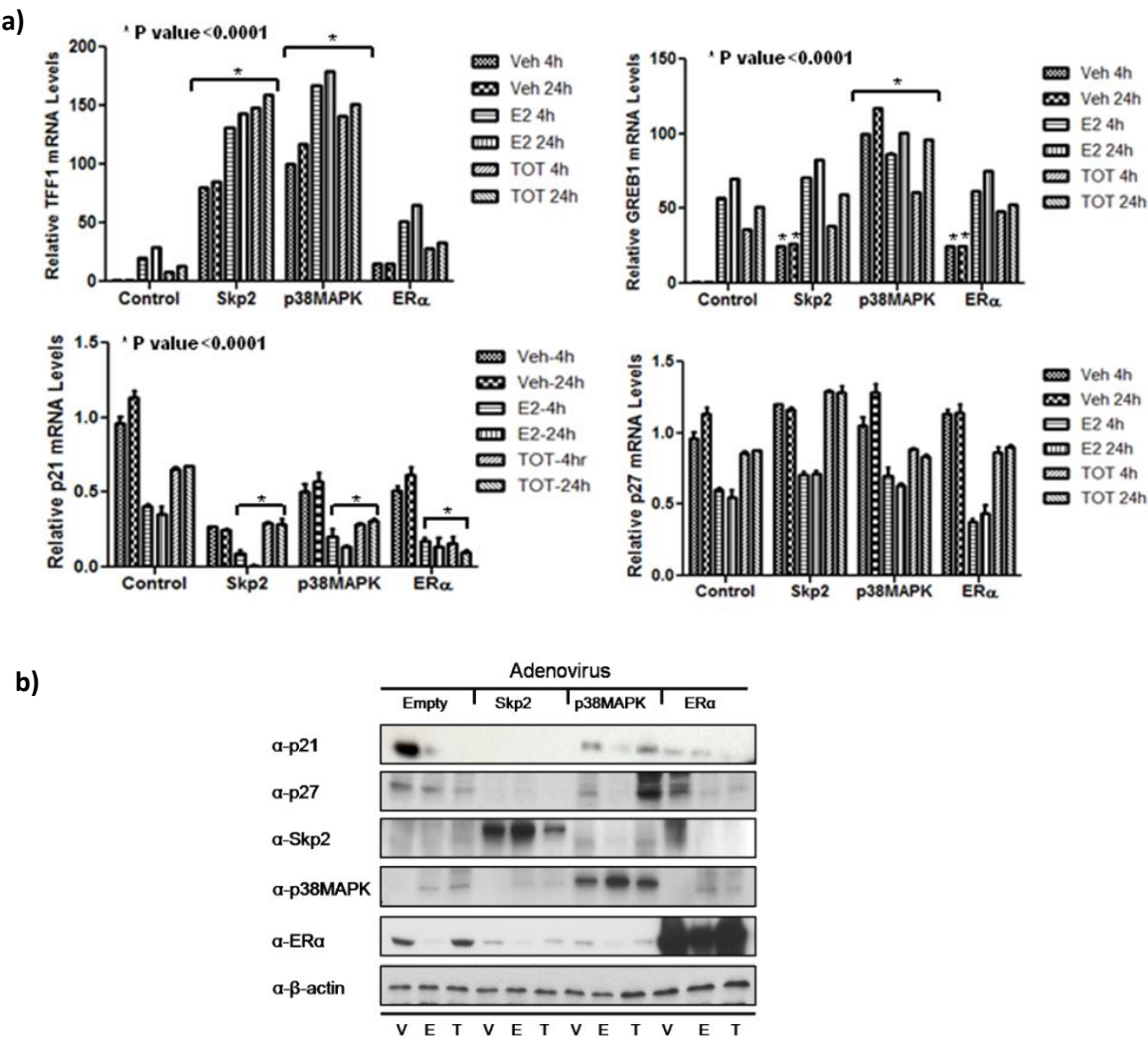
To address the apparent paradox between ER $\alpha$  degradation and S-phase entry of cells, we investigated the stimulus for p38MAPK targeted and Skp2 mediated ER $\alpha$  turnover. Although nuclear receptors are subject to ubiquitin-proteasome degradation both in the presence or absence of their cognate ligands (Nawaz et al., 1999a; Kopf et al., 2000; Lonard et al., 2000; Wijayaratne and McDonnell, 2001; Reid et al., 2002, 2003; Nonclercq et al., 2004; Tateishi et al., 2004; Callige et al., 2005; Dennis et al., 2005; Horner-Glister et al., 2005; Laio et al., 2005; Valley et al., 2005; Callige and Richard-Foy, 2006), the interaction and degradation of ER $\alpha$  by Skp2 was largely ligand independent. This observation prompted us to negate out the possibility for the role of hormone in facilitating Skp2 driven ER $\alpha$  turnover and hence cell cycle progression. However, since many critical regulators of growth have been suggested to demonstrate cyclical patterns of expression that oscillate through cell cycle phases, this struck us as a possibility for Skp2-ER $\alpha$  regulation (Lucas J et al., 1995; Fung TK et al., 2002; Galindo M et al., 2005; Koga S et al., 2007; Pyne S et al., 2009). Our work with synchronized MCF-7 cells showed enhanced Skp2 mediated trans-activation of both basal and estradiol induced ER $\alpha$  target gene expression, specifically in the G1/S and S-phase of the cells cycle, the stages that involved highest activation of p38MAPK leading to most of the Skp2 mediated regulation of ER $\alpha$  turnover. Therefore, Skp2 appears to induce both degradation and activation of ER $\alpha$ .

While many targets of estradiol-bound ER $\alpha$  showed elevated hormonal induction upon Skp2 over-expression, the most significant effect observed was on basal gene expression. This we believe could be due to cross-talk between Skp2 and various chromatin/histone modifying factors that could impact on the chromatin assembly in the promoters of ER $\alpha$  target genes. Another possibility is the degradation of negative regulators of ER $\alpha$  actions, such as Repressor of Estrogen receptor Activity (REA) (Umanskaya K et al., 2007) and Nkx3-1 (unpublished data by Bhatt S and Katzenellenbogen BS) by Skp2. Facilitation of

the recruitment of various co-activator proteins by Skp2 to ER $\alpha$  target gene promoters in the absence of hormone is another plausible mechanism that may need further investigation. These possibilities are strongly supported by the reports that suggest unliganded ER $\alpha$  is predominantly located in the nucleus as a monomer or a dimer (Aumais et al., 1997; Nathan et al., 1997; Ylikomi et al., 1998) and upon ligand binding, undergoes a conformational change that stabilizes the ER $\alpha$  dimer (Brzowski et al., 1997) and facilitates its recruitment to estrogen responsive promoters. We however, did not observe a major change in ER $\alpha$  recruitment to its target gene promoters upon Skp2 over-expression. Interestingly, we found both Skp2 and p38MAPK recruited to the promoters of ER $\alpha$  target genes (pS2, PgR, GREB1, p21 etc.), thereby supporting the hypothesis that it's the p38MAPK initiated and Skp2 mediated cycling of the unliganded and liganded receptor on estrogen responsive promoters that facilitates trans-activation of its target genes, which in turn facilitates the entry of cells into S-phase.

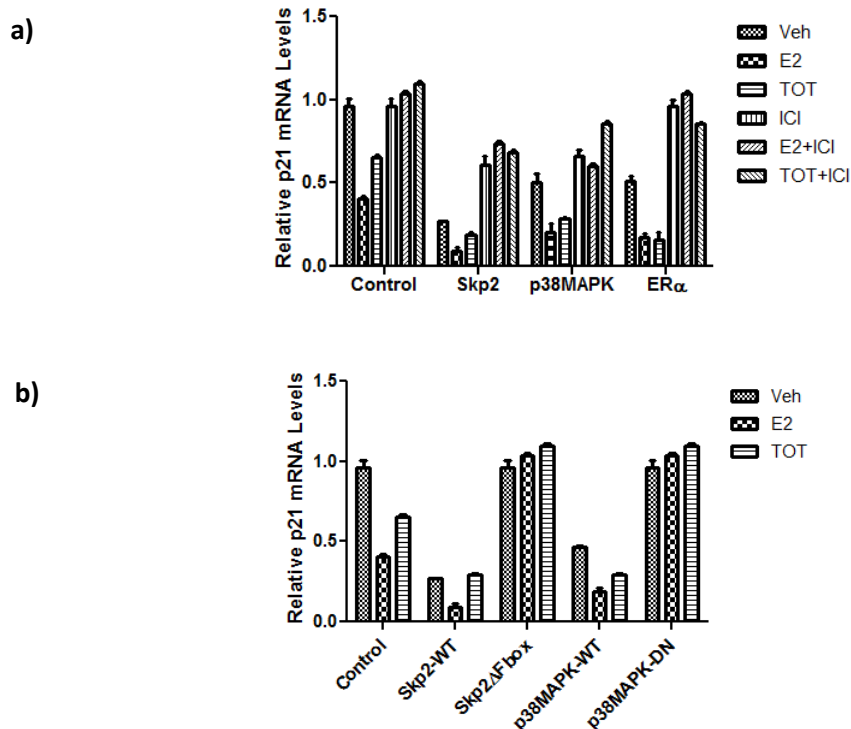
This enhanced gene trans-activation driven S-phase entry of cells due to increased Skp2 mediated ER $\alpha$  turnover may underlie the poorly understood phenomenon of more aggressive ER-negative breast tumors compared to ER-positive. Thus, our findings offer Skp2-p38MAPK mediated ER $\alpha$  turnover leading to potentiation of its trans-activation function as a novel pathway for drug targeting, inhibition of which may restore ER $\alpha$  expression in ER-negative tumors while still suppressing the proliferative effects of the receptor on cellular growth owing to dampened expression of its target genes.

FIGURES



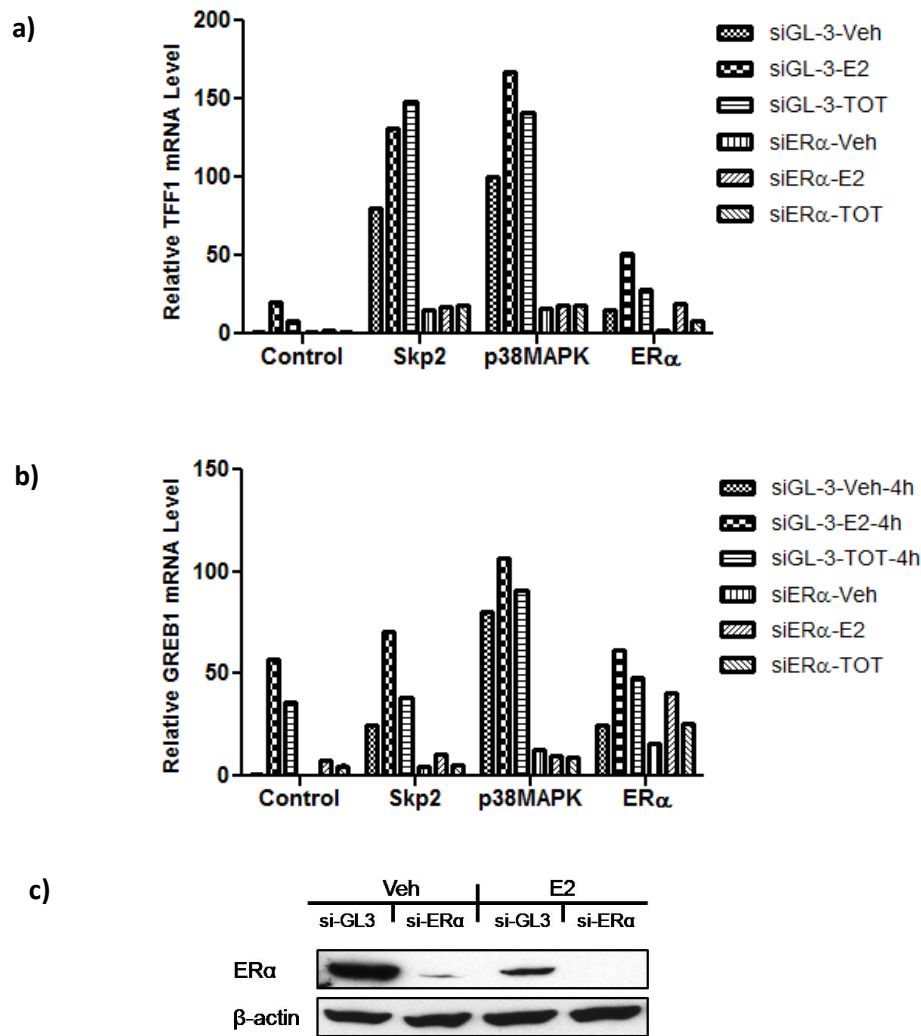
**Fig.4.7 Skp2 and p38MAPK Regulate ERα Target gene Expression in MCF-7 Cells**

a) MCF-7 cells weaned for 4 days were seeded in 6-well plates at 50-70% confluence, infected with control, Skp2, p38MAPK or ERα adenovirus and 24h post infection, treated with vehicle (0.1% EtOH), E2 (10nM) or Tamoxifen (1μM) for 4 and 24h before they were harvested and RNA extracted followed by Real-time PCR to monitor expression of various ERα target genes. Shown are relative mRNA expression levels for pS2, GREB1, p21 and p27 genes. b) Same as (a) but 24h ligand treatment samples subjected to protein analysis.



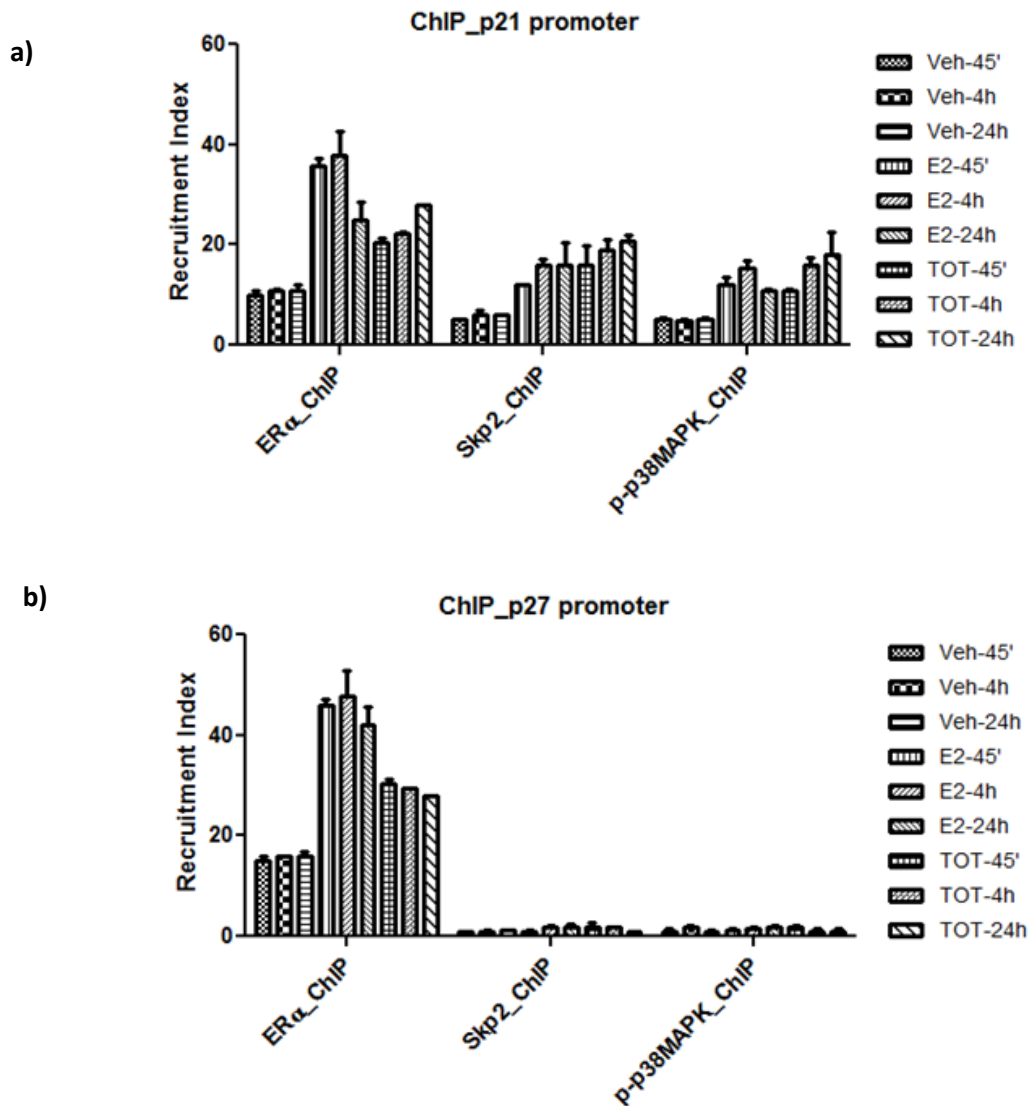
**Fig.4.8 Skp2 and p38MAPK Mediated Target Gene Regulation Is Through ERα and Requires the Ubiquitination and Kinase Activities of the Two Proteins**

a) MCF-7 cells grown as described in (a) were infected with either control, Skp2, p38MAPK or ERα adenovirus and 24h post infection, treated with vehicle, E2 (10nM), Tamoxifen (1μM), ICI (1μM) + E2 (10nM), ICI (1μM) + Tamoxifen (1μM) and ICI (1μM) for 4h before harvest. Real-time PCR was performed to monitor expression of ERα target gene, p21 while using 36B4 as internal control. b) MCF-7 cells grown as described in (a) were infected with control, Skp2-WT or Skp2ΔFbox mutant adenovirus, p38MAPK-WT or p38MAPK-DN adenovirus and 24h post infection, treated with vehicle, E2 (10nM), Tamoxifen (1μM), ICI (1μM), E2 (10nM) and ICI (1μM) or Tamoxifen (1μM) and ICI (1μM) for 4 and 24h before they were harvested followed by Real-time PCR to monitor expression of ERα target gene, p21 while using 36B4 as internal control. Other ERα target genes like pS2, GREB1, PgR were also monitored and showed similar result (data not shown).



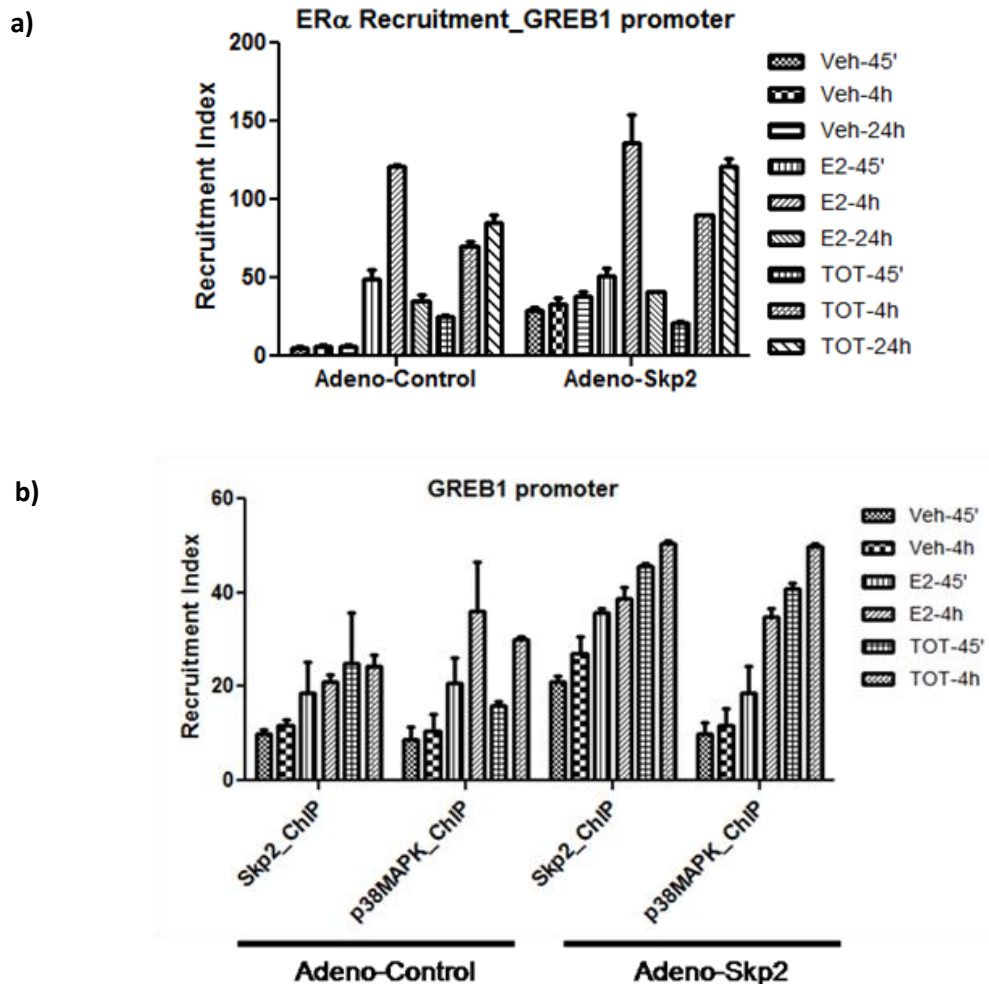
**Fig.4.9 ER $\alpha$  Knockdown Blocks the Transcriptional Regulation of ER-Target Genes by Skp2 or p38MAPK**

MCF-7 cells weaned for 4 days in 1X MEM w/o phenol red medium supplemented with 5% charcoal dextran stripped calf serum were seeded in 6-well plates at 50% confluence. The cells were subjected to siRNA against ER $\alpha$  for 24h followed by infection with control, Skp2, p38MAPK or ER $\alpha$  adenovirus for 24h and subsequent treatment with vehicle (0.1% EtOH), E2 (10nM) or Tamoxifen (1 $\mu$ M) for 4 before they were harvested and RNA extracted using Trizol reagent (Invitrogen) followed by Real-time PCR to monitor expression of ER $\alpha$  target genes, TFF1 (a) and GREB1 (b), using 36B4 as internal control. c) Western blot analysis: For analysis of ER $\alpha$  protein upon siRNA knockdown in MCF-7 cells.



**Fig.4.10 Skp2 and p38MAPK are Recruited to p21 not p27 Gene Promoter**

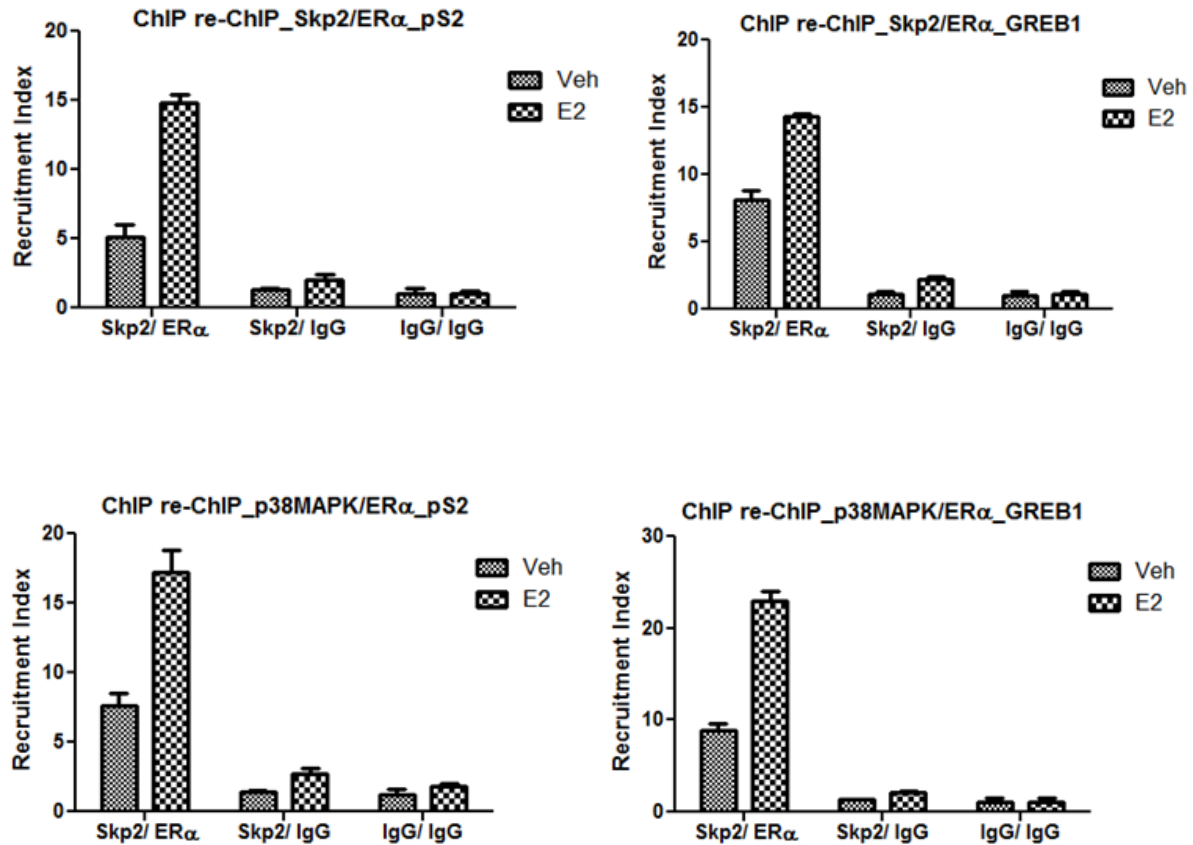
ChIP assays: MCF-7 cells weaned for 4 days in 1X MEM without phenol red medium and supplemented with 5% charcoal dextran stripped calf serum were seeded in 6-well plates at 50-70% confluence followed by 45', 4h or 24h of Vehicle, Estradiol (1nM) or Tamoxifen (1μM) treatment. Cells were subsequently harvested and washed with cold PBS (pH 7.2) to perform ChIP assays using specific ERα, Skp2 or p38MAPK antibody or IgG negative control antibody. Data are represented as recruitment index (Specific antibody signal/IgG signal ratio) and genes analyzed are p21 (a) and p27 (b).



**Fig.4.11 Skp2 Positively Impacts on ERα Recruitment to Target Gene Promoters**

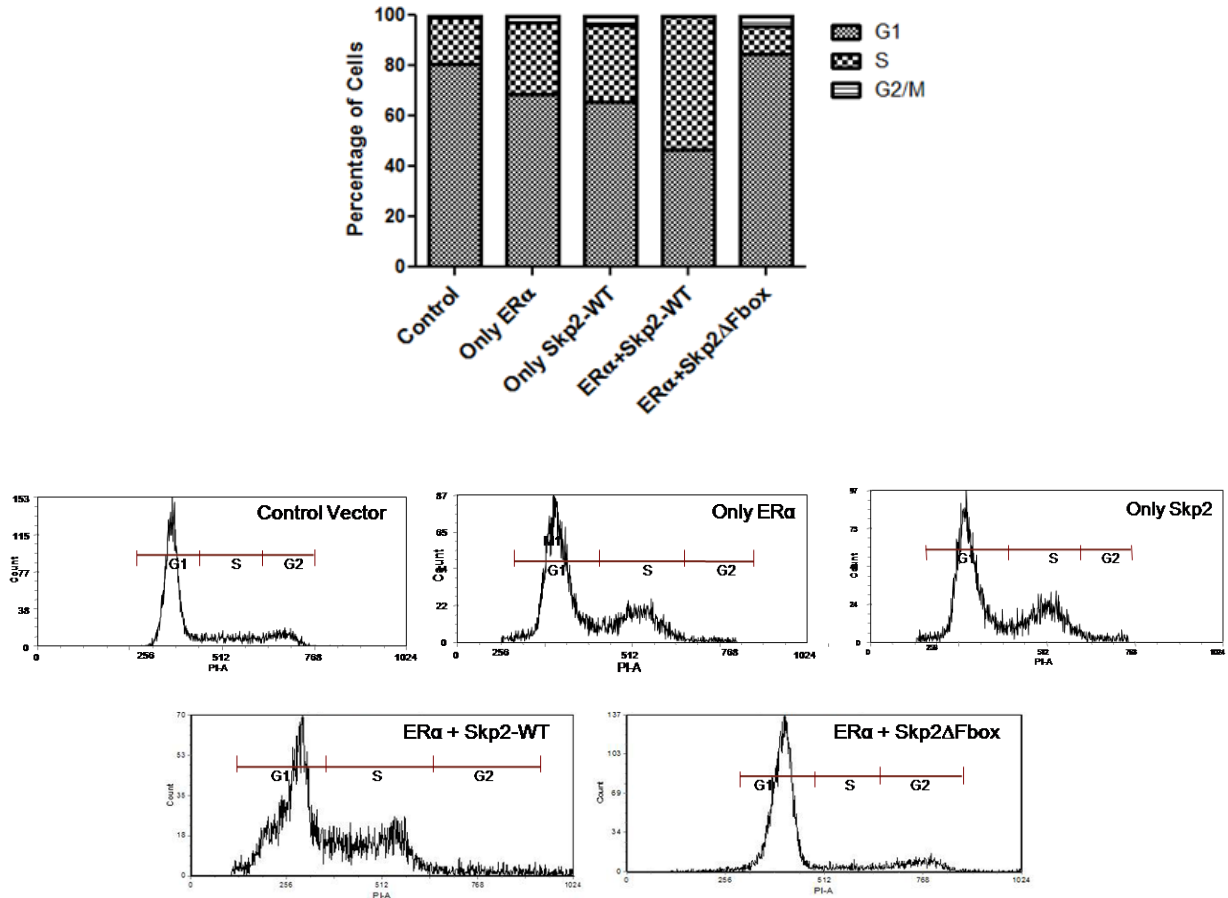
a) Chromatin Immunoprecipitation (ChIP) assays: MCF-7 cells were infected with either  $\beta$ -Gal (control) or Skp2 adenovirus for 24h followed by 45', 4h or 24h of Vehicle (0.1% EtOH), Estradiol (1nM) or Tamoxifen (1 $\mu$ M) treatment. Cells were harvested 48h post infection and washed with cold PBS (pH 7.2) to perform ChIP assays using either specific ER $\alpha$  antibody or IgG negative control antibody. Data are represented as recruitment index (ER $\alpha$  signal/IgG signal ratio) and gene analyzed is GREB1. Similar results obtained for TFF1 (data not shown). b) ChIP assays performed same as (a) using specific Skp2, p38MAPK antibody or IgG negative control antibody.





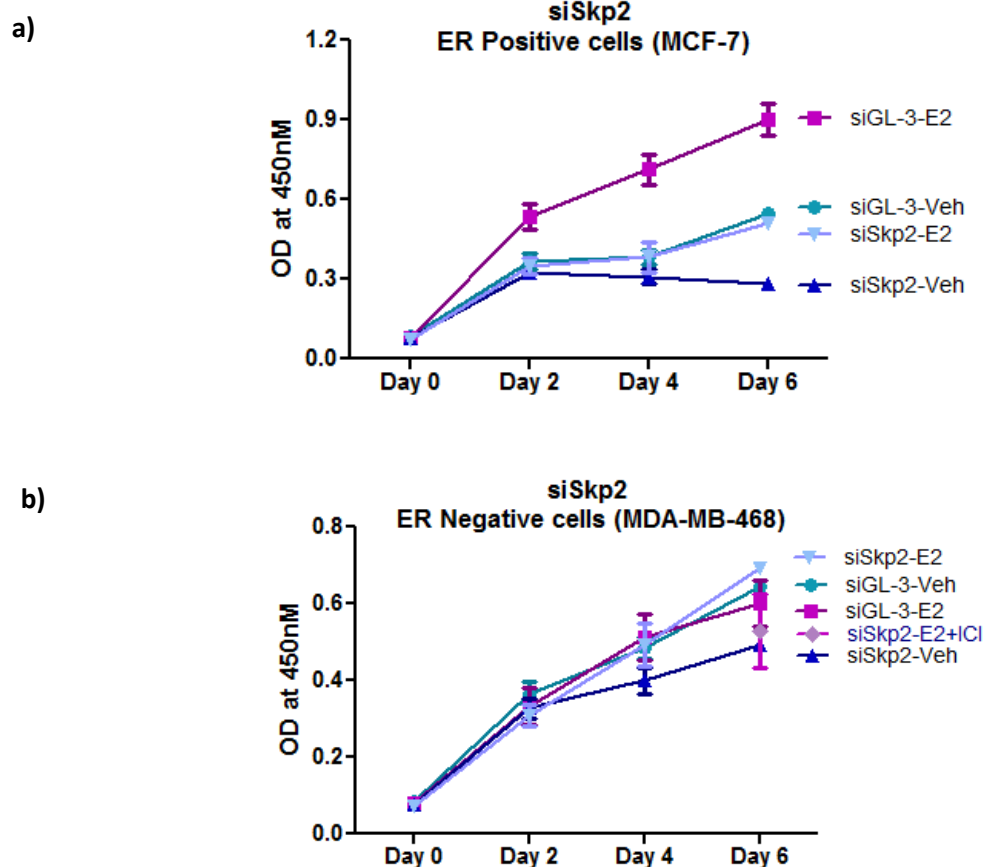
**Fig.4.12 ChIP re-ChIP: Skp2 and p38MAPK Co-exist with ERα in Transcriptional Complexes on the Promoters of ER-target Genes**

MCF-7 cells weaned in minimum essential medium (MEM) supplemented with 5% Charcoal Dextran (CD)-stripped calf serum for 4 days followed by treatment with vehicle (ethanol) or E2 (10nM) for 4h, were subjected to chromatin immunoprecipitation assay (ChIPs), as described in materials and methods, using specific antibody against Skp2, p38MAPK or IgG. Immunoprecipitated material from each ChIP pull-down was recovered with 10mM dithiothreitol in IP buffer at 37°C for 30min, diluted and subjected to a second round of immunoprecipitation using specific ERα antibody. Data are represented as recruitment index (Specific antibody signal/ IgG signal ratio) and genes analyzed are pS2 and GREB1.



**Fig.4.13 Skp2 Potentiates the Mitogenic Effects of Estradiol Through ERα**

MCF-7 cells were infected with adeno-β-gal (control adenovirus) or adeno-ERα alone or adeno-Skp2 alone or both adeno-ERα and adeno-Skp2 in combination. 12h post infection cells were treated with 10nM estradiol and harvested after 48h. Cells were stained with propidium iodide to know their DNA content and subsequently scanned by FACS analysis (BD Biosciences FACS-canto) to determine their cell cycle stage. b) FACS analysis graphs of experiment described in (a).



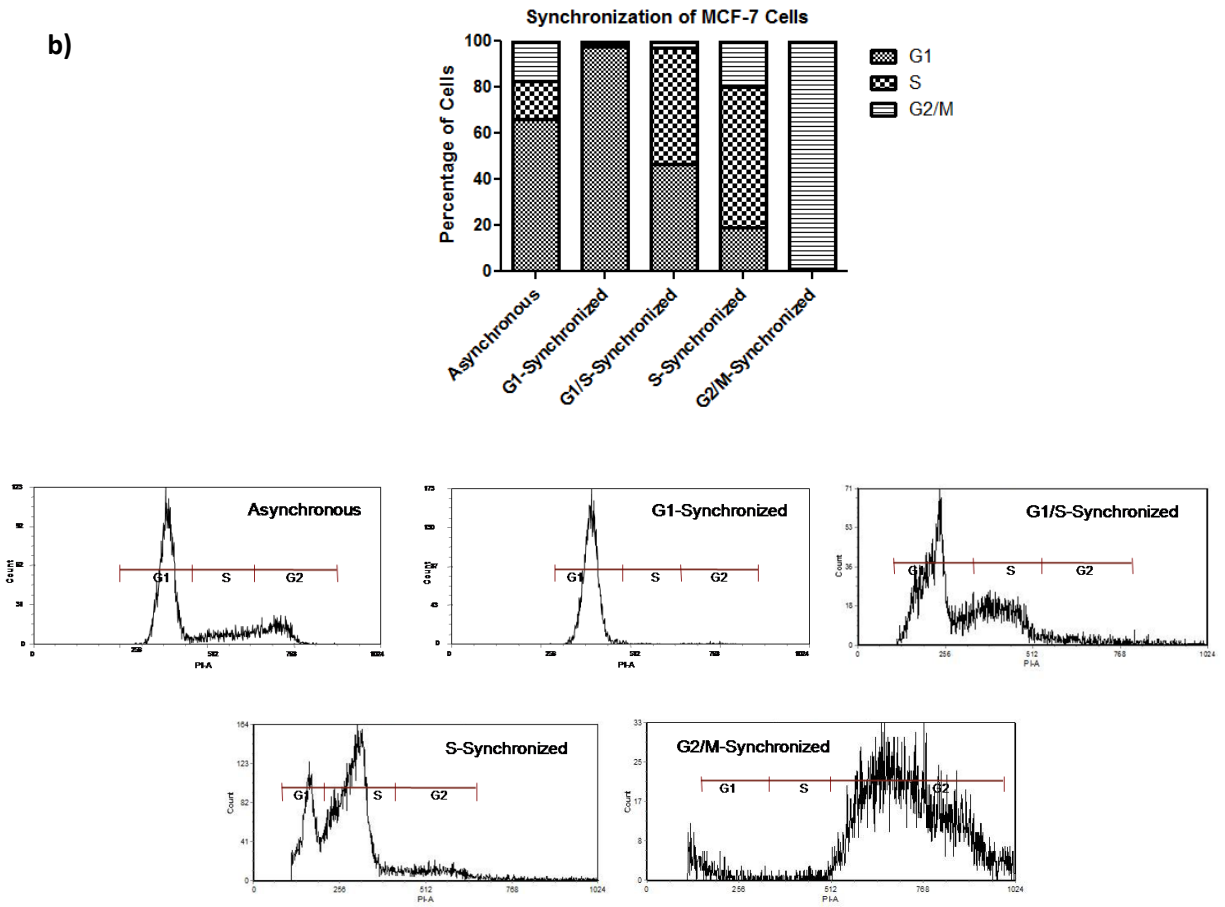
**Fig.4.14 Skp2 Potentiates the Mitogenic Effects of Estradiol Through ER $\alpha$**

(a) Growth assay was performed in ER $\alpha$  positive MCF-7 cells, in the presence of siRNA against Skp2 or non-targeting control followed by 0, 2, 4 and 6 day treatment with vehicle (0.1% EtOH), 10nM estradiol or 1 $\mu$ M tamoxifen followed by analysis of cell density at each time-point using WST reagent (Roche) by taking colorimetric reading in a luminometer. (b) Similar growth analysis as in (i) in ER $\alpha$  negative MDA-MB-468 cells.

a)

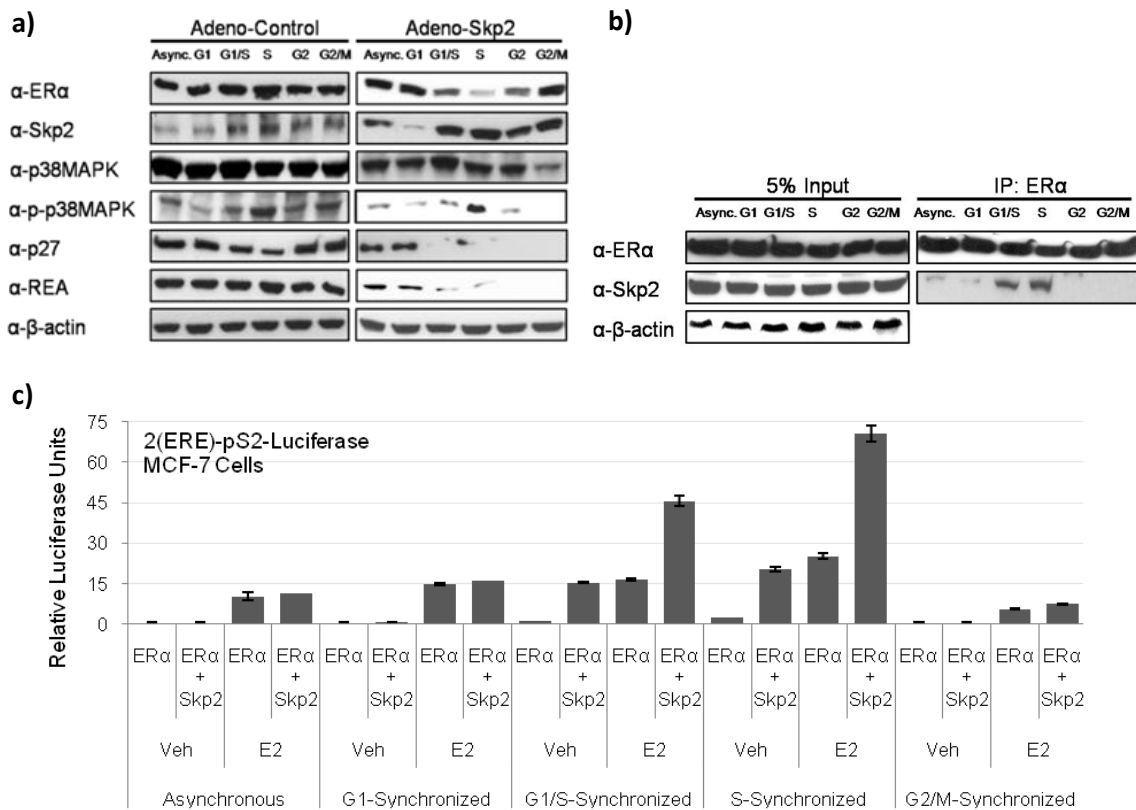
Time	24h	48h	72h	
Asynchronous				
G1	Serum starvation		Serum + Lovastatin	
G1/S	Serum starvation		Serum + Hydroxyurea	
S	Serum starvation		Serum + Hydroxyurea	Serum
G2	Serum + Hydroxyurea		Serum + Nocodazole	
G2/M	Serum + Hydroxyurea		Serum + Nocodazole	Serum

b)



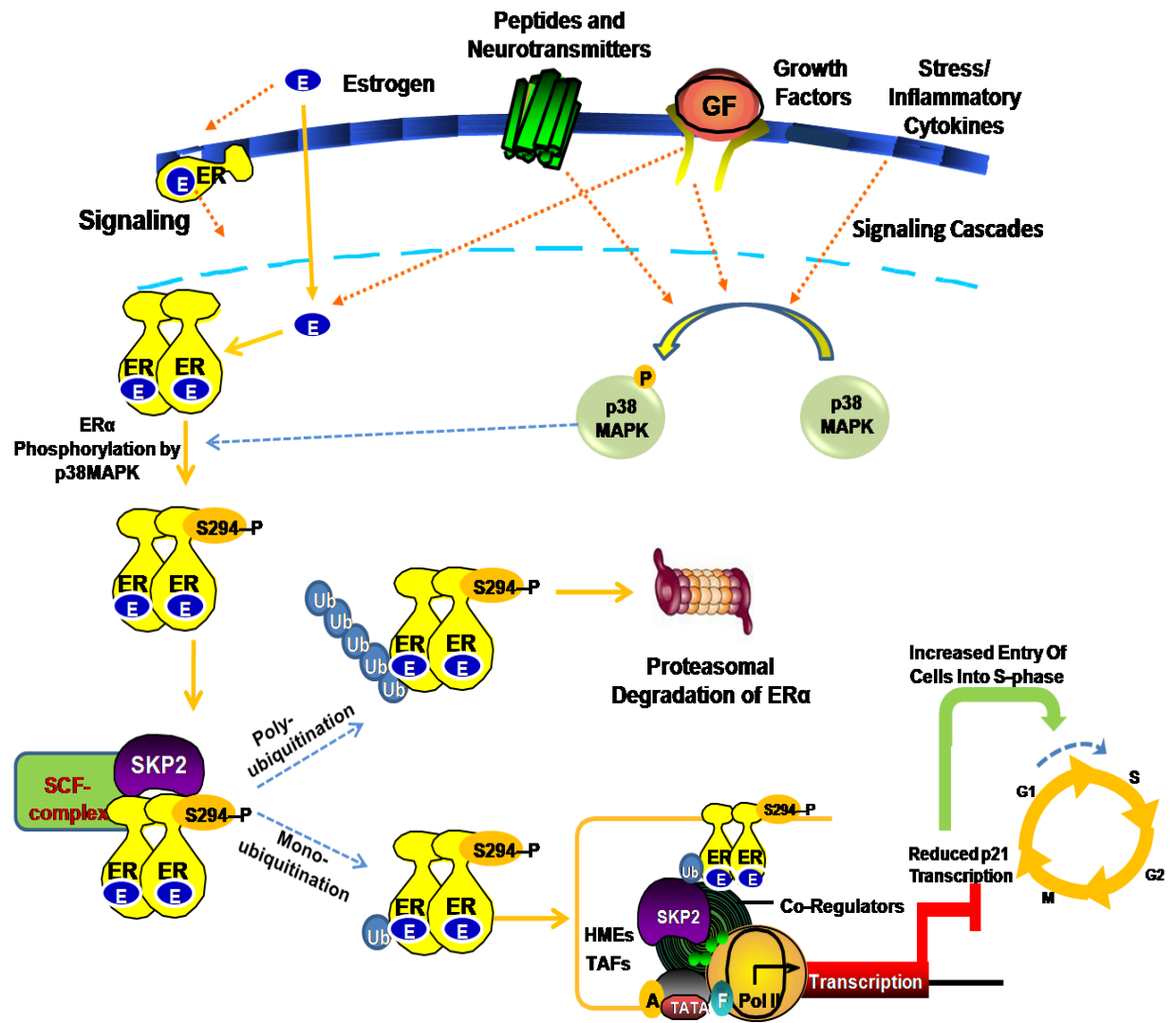
**Fig.4.15 Synchronization of MCF-7 Cells In Various Phases of the Cell Cycle**

a) Schematic of cell cycle synchronization of MCF-7 cells. b) FACS analysis of MCF-7 cells synchronized at various stages in the cell cycle (as described in materials and methods)



**Fig.4.16 Skp2 Interacts With and Ubiquitinates ERα Specifically in the G1/S and S-phases of the Cell Cycle**

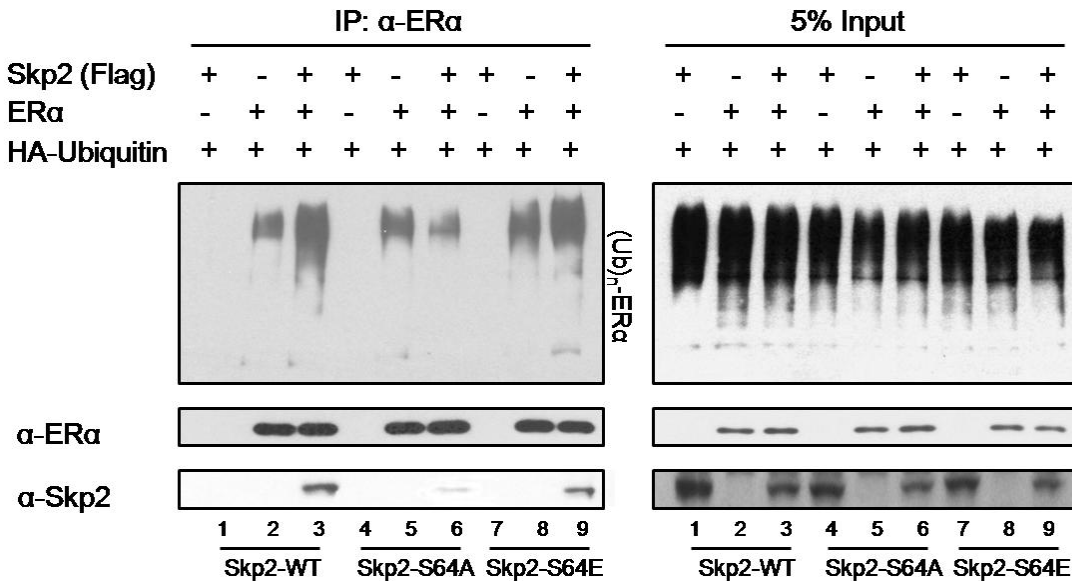
a) MCF-7 cells were synchronized and infected with adeno-Skp2, 24h later harvested for protein extraction and western analysis using anti-Skp2, anti-ERα, anti-p38MAPK and anti-phospho-p38MAPK antibodies. b) In a parallel experiment processed as in (a), cells were treated with proteasome inhibitor MG-132 (20μM) 5h before harvest and cell lysates were subjected to immunoprecipitation using specific antibody against ERα followed by SDS-PAGE and western blotting with anti-ERα and anti-Skp2. c) MCF-7 cells were synchronized at various stages in the cell cycle and transfected with 2ERE-pS2-Luciferase expression plasmid along with those for ERα and β-galactosidase. 6h post transfection cells were treated for 24h with either Vehicle (0.1% EtOH) or E2 (10nM) and thereafter harvested and frozen overnight at -80°C followed by measuring luciferase fluorescence intensity using a luminometer.



**Fig.4.17 MODEL**

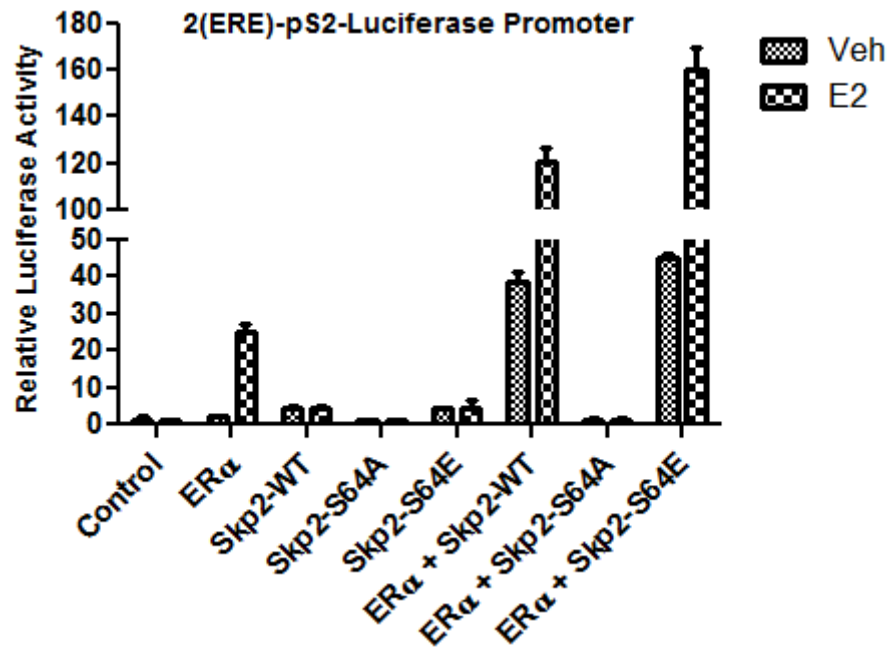
Schematic model highlighting the role of post translational modifications and their importance in regulating cellular turnover of ER $\alpha$  and as a consequence, cell cycle progression of breast cancer cells.

## SUPPLEMENTARY FIGURES



### ***S1. Skp2-S64A mutant does not Interact with or Ubiquitinate ER $\alpha$***

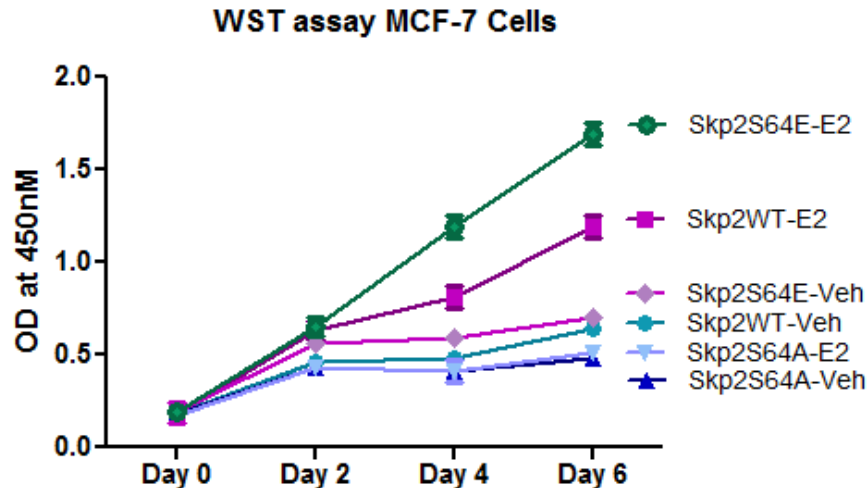
Cos-1 cells were subjected to ubiquitination assay by transfecting with Skp2-WT (wildtype Skp2), Skp2-S64A (Ser-64 replaced by alanine, phosphorylation defective mutant) or Skp2-S64E (Ser-64 replaced by glutamic acid, constitutively active phospho-mimic) along with ER $\alpha$  and HA-Ubiquitin (HA-Ub) or alone. Cells were treated with MG-132 (10 $\mu$ M) for 5h before harvest, 24h after transfection. Cell lysates were thereafter immunoprecipitated using anti-ER $\alpha$  antibody followed by western blotting with anti-ER $\alpha$ , anti-Skp2 and anti-HA-tag antibody to detect ubiquitinated ER $\alpha$ .



## ***S2. Skp2-S64A mutant is Ineffective in Modulating the Expression of ERα Target Genes***

Hec-1 cells were transfected with expression plasmid for 2ERE-pS2-Luciferase, ERα and Renilla Luciferase (internal control to assess transfection efficiency) along with Skp2-WT, Skp2-S64A or Skp2-S64E. 6h post transfection cells were treated for 24h with either Vehicle (0.1% EtOH) or E2 (10nM) and thereafter harvested and frozen overnight at -80°C followed by measuring luciferase fluorescence intensity using a luminometer.





### ***S3. Skp2-S64A Mutant Is Ineffective in Potentiating Estradiol Driven Growth of Breast Cancer Cells***

Growth assay was performed in ER $\alpha$  positive MCF-7 cells, with over-expression of wildtype Skp2, Skp2-S64A (mutant defective in binding and ubiquitinating ER $\alpha$ ) or Skp2-S64E (constitutively active mutant that binds and turn over ER $\alpha$ ) followed by 0, 2, 4 and 6 day treatment with vehicle (0.1% EtOH), 10nM estradiol or 1 $\mu$ M tamoxifen and analysis of cell density at each time-point using WST reagent (Roche) by taking colorimetric reading in a luminometer.

## **ACKNOWLEDGEMENTS**

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## **CHAPTER V- ROLE OF SKP2 IN REGULATING TAMOXIFEN-SPECIFIC INDUCTION OF OCT-3/4 IN BREAST CANCER CELLS**

### **ABSTRACT**

Tamoxifen has shown great success in the treatment of breast cancer, however, the problem of acquired tamoxifen resistance is still a challenge. Our study highlights novel players that together impact on ER $\alpha$  target gene expression via tamoxifen dependent mechanisms, inhibition of which we believe could make these tam-resistant tumors, amenable to therapy. Bioinformatics analysis of datasets from genome-wide mapping of ER $\alpha$  binding sites, in MCF-7 breast cancer cells, in the presence of estradiol or tamoxifen (Stunnenberg 2009) showed enrichment of Oct-3/4 and Nkx3-1 transcription factor binding sites in the promoters of target genes that recruit ER $\alpha$  preferentially or exclusively in the presence of tamoxifen (with >5 fold enrichment over estradiol). Interestingly, we found Oct-3/4 to be induced by tamoxifen, not estradiol, in an ER $\alpha$  dependent manner unlike other Oct isoforms also known to be expressed in MCF-7 cells. Upon further analysis we found Nkx3-1 to be regulating this tam-induction of Oct-3/4 by direct recruitment to the Nkx-response element in the Oct-3/4 promoter, as demonstrated by luciferase reporter assay with WT or Nkx-mut Oct-3/4 promoter.

In a parallel study we found Skp2, previously shown by our work to impact on ER $\alpha$  levels and function in breast cancer cells, to contribute significantly to the acquisition of tamoxifen resistance in MCF-7-tamr cells by regulating tamoxifen sensitivity of these cells. On further investigation we found Skp2 in complex with Nkx3-1, mediating its proteasomal degradation in a tamoxifen dependent manner. Interestingly, phosphorylation of Skp2 at Serine-64, a putative p38MAPK site validated by mass-spectrometry analysis and In-cell kinase assay, appeared essential for the ubiquitination of Nkx3-1 by the E-3 ligase. This phosphorylation that occurred preferentially in the presence of tamoxifen appeared

to be a likely mechanism underlying the tamoxifen preferential degradation of Nkx3-1 by Skp2. Furthermore, decreased recruitment of Nkx3-1 and hence phospho-RNA polII to Oct-3/4 promoter upon over-expression of Skp2 in the presence of tamoxifen, not estradiol implicated p38MAPK initiated and Skp2 mediated Nkx3-1 degradation as a likely mechanism for tamoxifen specific induction of Oct-3/4. Our work therefore, highlights a novel mechanism for the induction of Oct-3/4 upon tamoxifen treatment through p38MAPK catalyzed phosphorylation of Skp2 at Ser-64 and subsequent degradation of Nkx3-1.

## INTRODUCTION

The anti-estrogen tamoxifen which acts by inhibiting the growth stimulatory effects of estradiol largely by blocking gene expression through estradiol bound ER $\alpha$  has been widely used for decades for treating patients with ER $\alpha$  positive or hormone dependent breast cancer. Despite its obvious benefits in containing breast cancer, in as high as 40% of the patients receiving adjuvant tamoxifen there is an eventual relapse of the disease largely due to intrinsic (de novo) or acquired resistance to tamoxifen, underlying mechanism for which is rather poorly understood. Elucidating the molecular basis underlying the agonistic effects of tamoxifen on cellular growth and hence the issue of “acquired tamoxifen resistance” would require thorough understanding of the unique role of tamoxifen in regulating ER $\alpha$  target gene expression independent of estradiol. Although there have been elaborate studies with genome-wide analysis of ER $\alpha$  binding sites within chromosomal loci in the presence of estradiol or tamoxifen, information on gene regulation unique to tamoxifen bound ER $\alpha$  is not fully understood. This is where the importance of our work stems as it offers a unique mechanism for this rather arcane phenomenon thereby offering targets for new drug discovery to block tamoxifen resistance and making tam-resistant tumors amenable to therapy.

We therefore wanted to investigate the molecular basis for anti-estrogen to pro-estrogen switch of tamoxifen action in order to understand the underlying mechanism for tamoxifen resistance in breast cancer. To this end, we performed bioinformatics analysis on the recently elucidated genome-wide maps for ER $\alpha$  binding sites across distinct chromosomal loci in the presence of estradiol or tamoxifen in MCF-7 breast cancer cells (Brown 2006, Stunnenberg 2009) and found enrichment of Oct-3/4 and Nkx3-1 transcription factor binding sites in the promoters of target genes that recruited ER $\alpha$  preferentially or exclusively in the presence of Tamoxifen (with >5 fold enrichment over estradiol). Interestingly, we found Oct-3/4 to be induced by tamoxifen, not estradiol, in an ER $\alpha$  dependent manner and this tamoxifen induction required Nkx3-1 as confirmed by luciferase assay in MCF-7 cells with WT or Nkx-mut Oct-3/4 promoter, with abrogated Nkx-binding site, fused to 2(ERE)-pS2-luciferase gene. Interestingly, we observed much elevated Oct-3/4 protein levels while reduced Nkx3-1 protein in MCF-7-tamr derivatives compared to MCF-7. Growth assays in MCF-7-tamr cells in the presence of tamoxifen parentals extended further support for the role of these factors in regulating tamoxifen sensitivity and hence acquisition of tamoxifen resistance by these cells (see chapter VI).

In a parallel study we found, Skp2, an E-3 ubiquitin ligase and also a major regulator of cell cycle regulates ER $\alpha$  levels and function in breast cancer cells. To our great surprise then Skp2 was found to mediate ubiquitin proteasomal degradation of ER $\alpha$  while at the same time acting as a co-activator to transcriptional regulation via ER $\alpha$ . Since the effects of tamoxifen are primarily mediated through the ER and the degree of ER expression is a strong predictor of response to tamoxifen we investigated the role of Skp2 in regulating the sensitivity of MCF-7-tamr cells to tamoxifen. Skp2 was indeed found to regulate tamoxifen sensitivity of these cells largely by interacting with and ubiquitinating Nkx3-1 in a tamoxifen dependent manner, upon activation of Skp2 through p38MAPK dependent phosphorylation, to mediate tam-specific induction of Oct-3/4, a factor highlighted in our work to have binding sites enriched in

tamoxifen preferential ER $\alpha$  target gene promoters, and documented to be imperative in the regulation of tamoxifen dependent growth of these cells by Skp2 (see chapter VI).

## **EXPERIMENTAL PROCEDURES**

### ***Antibodies-***

Anti-Skp2 (N-19, H-435, A-2), anti-ER $\alpha$  (HC-20, F-10), anti-Ub (P4D1), anti-HA tag (F-7, Y-11), anti-Oct-1, 2, 3/4 and 11 were purchased from Santa Cruz Biotechnology. Anti-Myc tag (2272, 2276), anti-p38MAPK (9212, 9228), anti-phospho (Thr180/Tyr182) p38MAPK (9211, 9216) were from Cell Signaling and anti-flag rabbit and mouse and anti-phosphoserine antibody conjugated agarose beads were purchased from Sigma. Horseradish peroxidase (HRP)-conjugated donkey anti-goat, donkey anti-mouse and donkey anti-rabbit IgG secondary antibodies were from Santa Cruz Biotechnology.

### ***Cell Culture and Transfections-***

Cos-1 cells were purchased from American Type Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) [Hyclone] supplemented with 10% Fetal Bovine Serum (FBS) [Hyclone, Logan, UT], 100 units per ml of Penicillin and 50  $\mu$ g/ml of Streptomycin at 37°C in 5% CO<sub>2</sub> with humidified air. MCF-7 cells were maintained in growth medium containing Minimum Essential Medium (MEM)-with phenol red (SIGMA) supplemented with 5% heat inactivated Calf Serum (CS) [Hyclone, Logan, UT], 100 units per ml of Penicillin and 50  $\mu$ g/ml of Streptomycin at 37°C in non-CO<sub>2</sub> incubator or treatment medium containing Minimum Essential Medium (MEM)-without phenol red supplemented with 5% Charcoal dextran stripped Calf Serum (CS) at 37°C under 5%CO<sub>2</sub> in humidified air. All transient plasmid transfections were performed using LipofectAMINETM 2000 Reagent from Invitrogen according to manufacturer's instructions. siSkp2 transfection was performed using Lipitoid reagent kindly provided

by Dr. Ronald Zuckermann at the Molecular Foundry, Lawrence Berkeley National Laboratory. All other siRNA transfections were performed using Dharmafect transfection reagent from Dharmacon.

### ***Plasmids-***

Skp2 cDNA was purchased by Open Biosystems and cloned into pcDNA3-Flag expression vector. Various Skp2 deletion mutants were generated from this full-length construct by PCR based subcloning. ER $\alpha$  was cloned into either pcDNA3-Flag expression vector or pcDNA3.1-Myc/His expression vector from Invitrogen. Site directed mutagenesis kit from Stratagene was used to introduce various Ser to Ala or Glu point mutations in pcDNA3-Flag-Skp2 (full-length). pCMV-HA-Ubiquitin was generated by subcloning from GST-Ubiquitin expression vector (Addgene plasmid 10861) originally generated by Peter Howley. pMEV-2HA-p38MAPK-WT (wildtype) and DN (dominant negative) mutant were purchased from Biomyx Technology. Adenoviral vectors encoding Skp2-WT or  $\Delta$ F-box mutant were constructed by cloning relevant sequences into pAdTrack vector purchased from Stratagene. Details for generation of any of the above mentioned expression vector, is available upon request.

### ***Silencing by Small Interfering RNA (siRNA)-***

MCF-7 cells seeded onto 6-well plates at a confluence of 40-60% were transfected with siRNA duplexes, siSkp2 (Qiagen), sip38MAPK (Dharmacon), siNkx3-1 (Dharmacon), Lentiviral siOct-1 (Santa Cruz), Lentiviral siOct-2 (Santa Cruz), Lentiviral siOct-3/4 (Santa Cruz), Lentiviral siOct-11 (Santa Cruz), or non-targeting control siGL-3 (Dharmacon or Qiagen) and Lentiviral siGL-3 (Santa Cruz). siSkp2 transfection was performed using Lipitoid reagent kindly provided by Dr. Ronald Zuckermann at the Molecular Foundry, Lawrence Berkeley National Laboratory. All other siRNA transfections were performed using Dharmafect transfection reagent from Dharmacon.



### ***Adenovirus Construction and Infection for Over-expression of Endogenous Factors-***

cDNAs for the genes of interest, namely Skp2-WT, Skp2 $\Delta$ Fbox, p38MAPK-WT, p38MAPK-DN (dominant negative), ER $\alpha$ , Oct-1, Oct-3/4, Oct-11, were first cloned into pAdTrack-CMV shuttle vector from Stratagene or pcDNA3.1-CMV vector using PCR based subcloning. DNA from positive clones of pAdTrack-CMV constructs was linearized by digestion with PmeI and electroporated into electrocompetent BJ5183 cells (Stratagene) already containing plasmid-P1 for the recombination event to occur. Recombined DNA was thereafter extracted and transfected into AD293 cells for adenovirus preparation. After rounds of amplification the virus obtained was then purified by CsCl density gradient centrifugation and characterized for its titre and ability to over-express the protein of interest. MCF-7 cells seeded onto 6-well plates at a confluence of 40-60% were thereafter infected with this virus or an equal MOI (Multiplicity of Infection) of a non target control virus followed by ligand treatment (if required). Cells were harvested and processed for RNA and/or protein extraction as per requirement. In some experiments an alternative strategy (Weigel et al.) was used to introduce genes of interest cloned in regular pCMV-vectors into MCF-7 cells along with a poly-lysine coated adenovirus that was able to over-express the protein(s) of interest, namely Nkx3-1, NFATC4, by at least 2-3 folds.

### ***Luciferase Reporter Gene Assay-***

MCF-7 or Hec-1 cells were transfected with plasmids of interest using LipofectAMINE 2000 following manufacturer's protocol. The dual luciferase assay system used was purchased from Promega Corp. (Madison, WI). Promoters for Oct-3/4 and NFATC4 genes were cloned into 2(ERE)-pS2-Luciferase gene-pCMV plasmid upstream of the pS2 gene and transfected along with Renilla luciferase gene plasmid (Clontech, Palo Alto, CA). Site-directed mutagenesis was performed to abrogate Nkx and Oct transcription factor binding sites in Oct-3/4 and NFATC4 promoters respectively using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. The mutagenesis

sense and antisense primers were designed using the Stratagene web site. The plasmids were then sequenced to confirm the mutation of the desired site. Luciferase values were normalized using renilla luciferase gene as internal control for transfection efficiency. The amounts of plasmids transfected were: 1µg of the promoter constructs, 50 ng of Renilla luciferase construct, and 100 ng of ERα construct. Every experiment was performed at least three independent times.

#### ***Gene expression analysis by Quantitative Real-time PCR-***

MCF-7 cells seeded onto 6-well plates at a confluence of 40-60% were either siRNA transfected to silence the gene of interest or infected with adenovirus to over-express the gene of interest followed by treatment with ligand if necessary. RNA was isolated using Trizol reagent (Invitrogen) following manufacturer's protocol. The mRNA for genes of interest, namely Oct-1, Oct-2, Oct-3/4, Oct-11, Nkx3-1, NFATC4 was quantitated by SYBr Green-based reverse transcriptase-PCR (RT-PCR) using the ABI7600ht Sequence Detection System (Applied Biosciences). All mRNA quantities were normalized against 36B4 and primer sequences are available upon request.

#### ***Chromatin Immunoprecipitation (ChIP) and sequential ChIP (reChIP) assays-***

Chromatin Immunoprecipitation assays (ChIPs) were performed with minor modifications as described in Metivier et al. (Gannon 2003). MCF-7 cells were weaned in minimum essential medium (MEM) supplemented with 5% Charcoal Dextran (CD)-stripped calf serum for 4 days followed by treatment with vehicle (ethanol), E2 (10nM) or Tamoxifen (1µM) for 45', 4h or 24h. In some experiments this ligand treatment was preceded by 48h long infection with Adeno-Skp2-WT or Adeno-Skp2ΔFbox, Adeno-p38MAPK-WT or Adeno-p38MAPK-DN, or equal MOI of a control adenovirus. Antibodies used for pull-downs were purchased from Santa Cruz Biotechnology [ERα (HC-20), Skp2 (N-19), Nkx3-1 (N-15), RNA polymerase II (N-20), Oct-3/4 (C-10 and N-19)], Abcam [RNA polymerase II (ab817), RNA polymerase II phospho-Ser2 (ab5095), RNA polymerase II phospho-Ser5 (ab5131)] and Bethyl Laboratories Inc.

[p38MAPK (A310-212A)]. The DNA isolated was subjected to quantitative real-time PCR using gene specific primers (sequences available upon request) while using 36B4 as internal control (and measuring total input DNA in every sample) and a recruitment index was calculated (ratio between specific antibody signal over IgG signal). ChIP/reChIP experiments were done following the same ChIP protocol. After the first pull-down, immunoprecipitated material was recovered with 10mM dithiothreitol in IP buffer at 37°C for 30min, diluted and subjected to a second round of immuno-precipitation.

#### ***Co-Immunoprecipitation assay-***

Cos-1 cells were seeded onto 6-well plates at a confluence of 80-90% and transfected with plasmids of interest. 24h post transfection cells were harvested, washed with ice-cold PBS and solubilized with 0.5ml CoIP buffer (50mM Tris pH7.5, 150mM NaCl, 0.1% Nonidet P-40, 5mM EDTA, 0.1mM sodium orthovanadate, 1mM dithiothreitol (DTT), 1mM PMSF and 1X Protease inhibitor cocktail from Roche). Immunoprecipitation was carried out using 2-5ug specific antibody by incubation at 4°C overnight with gentle rotation. The precipitated proteins were collected by incubating with Protein-G sepharose (GE Healthcare) for 2h at 4°C with gentle rotation. After low speed centrifugation to remove the supernatant, the agarose was washed with CoIP buffer, boiled in SDS sample buffer and subjected to SDS-PAGE and Western blotting. In case of MCF-7 cells, endogenous proteins were Immunoprecipitated from lysates obtained from 10cm<sup>2</sup> plates using similar protocol as mentioned above with or without the involvement of transfections or adenoviral infections for exogenous over-expression of factors.

#### ***Ubiquitination assay-***

Cos-1 cells transfected with Nkx3-1 and other plasmids were pretreated with 10μM MG-132 (Calbiochem, La Jolla, CA) for 5h to block proteasome activity, before harvesting them 24h post transfection. Following harvest, cells were lysed in buffer containing 50mM Tris pH 7.5, 150mM NaCl, 10% glycerol, 5mM EDTA, 1% Nonidet P-40, 0.1mM sodium orthovanadate, 1mM dithiothreitol (DTT),

1mM PMSF and 1X Protease inhibitor cocktail from Roche. Immunoprecipitation was carried using 3µg ERα antibody by incubation at 4°C overnight with gentle rotation. The precipitated proteins were collected by incubating with Protein-G sepharose (GE Healthcare) for 2h at 4°C with gentle rotation. After low speed centrifugation to remove the supernatant, the agarose was washed with CoIP buffer, boiled in SDS sample buffer and subjected to SDS-PAGE and Western blotting with anti-HA antibody (Santa Cruz).

#### ***In Cell Kinase assay-***

To perform in cell kinase assay, Cos-1 cells were transfected with Flag-Skp2-WT or Flag-Skp2-S64A alone or along with HA-p38MAPK-WT or dominant negative kinase dead HA-p38MAPK-DN mutant with either no treatment or treatment with vehicle (ethanol), E2 (1nM) or tamoxifen (1µM) 6h post transfection. Cells were harvested 24h post ligand treatment and subjected to immunoprecipitation in CoIP buffer, as described earlier, using phosphoserine-antibody conjugated agarose beads (Sigma) followed by SDS-PAGE separation and western blot analysis using Skp2 and p38MAPK antibodies. Phosphorylation event was detected by an upward shift of the Skp2 band in the presence of the wildtype kinase, not the kinase dead mutant.

#### ***Cell Proliferation assay-***

WST-1 cell proliferation assay is a colorimetric assay based on the cleavage of a tetrazolium salt, MTS, by mitochondrial dehydrogenases to form formazan in viable cells following the addition of WST-1, which can be detected by measuring absorbance at 450nm, which in turn is used as a measure of cell proliferation and viability. MCF-7-tamr cells were counted using a hemocytometer and seeded at 50,000 cells/well in a 12-well plate. Seeded cells were incubated overnight at 37°C with 5% CO<sub>2</sub> in a humidified atmosphere and next day treated with ligand, vehicle (ethanol), E2 (1nM) or Tamoxifen (1µM) and harvested after 0, 2, 4 and 6 days of treatment. At the time of harvest media was aspirated and 500µl of

WST-1 reagent diluted 1:10 with 1XMEM supplemented with 5% CD stripped-Calf serum was added to each well. Incubation was continued for an additional 30min at 37°C with 5% CO<sub>2</sub> in a humidified atmosphere before absorbance was measured at 450nm versus a 650nm reference to compare the experimental samples to control, in Spectra MAX 190 microplate reader with SoftMax Pro software from Molecular Devices. Results were analyzed by exporting the data to MS Excel and expressing the mean  $\pm$  SD for each sample, while every sample was run in triplicate.

### ***Mass-spectrometry analysis-***

ER $\alpha$  was expressed alone or along with Flag-Skp2 or p38MAPK in MCF-7 cells by adenoviral infection in the presence of 5 $\mu$ M of MG132 to block proteasomal degradation and 48h later cells were harvested in CoIP buffer followed by immunoprecipitation using a specific anti-ER $\alpha$  antibody and several rounds of washings to purify ER $\alpha$  to near 90% homogeneity. Purified protein extracts were subjected to SDS-PAGE separation followed by visualization of ER $\alpha$  band by coomassie blue staining (A) or tandem mass spectrometry MS/MS analysis (B). A) ER $\alpha$  and IgG heavy chain bands are indicated in the coomassie stained gel picture. B) A Skp2 peptide modified with phosphorylation at serine-64 (57SNLGHPESpPPRKRLK71) was identified in a MS2 spectrum and confirmed by subsequent MS3 scan. S\*: phosphorylated serine-64; S#: serine-64 with loss of water.

Gel slices corresponding to ER $\alpha$  band (shown in A) were excised, destained in 50% acetonitrile in 25 mM NH<sub>4</sub>HCO<sub>3</sub> pH 8.4, lyophilized, and digested with 20ng/ $\mu$ L of trypsin in 25 mM NH<sub>4</sub>HCO<sub>3</sub> pH 8.4 (Promega, Madison, WI) overnight at 37 °C. The tryptic peptides were extracted from gel slices using 70% acetonitrile containing 5% formic acid. The peptide solution was lyophilized and reconstituted in 0.1% formic acid prior to nanoflow reversed-phase liquid chromatography (nanoRPLC) mass spectrometry analysis. NanoRPLC 4 columns were slurry-packed with 5  $\mu$ m, 300 Å pore size C-18 silica-bonded stationary reverse-phase particles (Jupiter, Phenomenex, Torrance, CA) in a 75  $\mu$ m i.d. x 10 cm

fused silica capillary (Polymicro Technologies, Phoenix, AZ) with a flame pulled tip. The column was connected to an Agilent 1100 nanoLC system (Agilent Technologies, Palo Alto, CA) and coupled to a linear ion-trap (LIT) mass spectrometer operated with Xcalibur 1.4 SR1 software (LTQ, ThermoElectron, San Jose, CA). The samples were injected onto the column and the peptides eluted using a gradient of mobile phase A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) under the following conditions: 2% B at 500 nL/min in 20 min; a linear increase of 2-42% B at 250 nL/min in 40 min; 42-98% B at 250 nL/min in 10 min; and 98% B at 500 nL/min for 18 min. The LTQ was operated in a data-dependent mode in which the five most abundant peptide molecular ions in every MS scan were sequentially selected for fragmentation and acquisition of MS2 spectra. The acquisition of neutral loss MS3 spectra was triggered when a neutral loss of phosphoric acid ( $\text{H}_3\text{PO}_4$ ) was detected with a loss of  $m/z$  98, 49, or 32.7 Da among the five peptide fragment ions in the MS2 scans. The normalized collision energy was set at 35% for both MS2 and MS3 collision-induced dissociation (CID). Dynamic exclusion was applied to minimize repeated selection of peptides previously selected for CID. The ion source capillary voltage and temperature were set at 45 V and 160 °C, respectively. The electrospray voltage was set at 1.6 kV.

Tandem mass spectra were searched against the human Skp2 protein sequence using SEQUEST software (ThermoFinnigan, San Jose, CA) operating on a 40-node Beowulf cluster. Fully tryptic cleavage constraints and two missed cleavage sites were applied for search. Phosphorylation of serine, threonine and tyrosine (+79.9663 Da) and oxidation of methionine (+15.9949 Da) residues were included as dynamic modification when searching the MS2 spectra, whereas phosphorylation of serine, threonine and tyrosine (+79.9663 Da), loss of water (-18.0106 Da) and oxidation of methionine (+15.9949 Da) residues were set dynamic modification when searching the MS3 spectra. The SEQUEST filtering criteria for initial identification of peptides were cross correlation (Xcorr) scores of 1.9 for  $[\text{M} + \text{H}]^+ 1+$ , 2.2 for  $[\text{M} + 2\text{H}]^{2+}$ , 3.1 for  $[\text{M} + 3\text{H}]^{3+}$  ions, and a minimum delta correlation ( $\Delta\text{Cn}$ ) of 0.08. The identified

phospho-peptides were further examined by manually inspecting the MS2 and MS3 spectra to confirm the identification of correct peptide sequence and modifications.

### ***Computational Motif analysis-***

De novo motif analysis was performed as previously described (Glass CK, Cell 2005). Sequences corresponding to the ER $\alpha$  binding sites, 200bp centered on ER binding peak, were extracted from the March 2006 human genome assembly Hg18. Binding sites were divided into two sets: E2 preferential (>5x tags in E2 state compared to Tam state) and Tot preferential (>5x tags in Tot state compared to E2 state). An exhaustive search for all n-mers ( $6 < n < 13$ ) was performed and each n-mer was scored for its enrichment in the E2 preferential binding sites using the hyper-geometric distribution relative to the Tot binding site sequences. This was also done with the Tot preferential peaks using the E2 binding site sequences as background. This was performed to mask out the EREs and common elements and should identify motifs that are specifically in a subset. The top 500 n-mers with a p-value less than 0.01 were then clustered together and used to create position-specific probability matrices. The matrices were then further optimized to discriminate motifs between E2 preferential and TOT preferential peaks.

## **RESULTS AND EXPERIMENTAL FINDINGS**

### ***Oct-3/4 Is Induced Specifically by Tamoxifen, not Estradiol, and its Binding sites Enriched in the Promoters of Tam-bound ER $\alpha$ Target Genes***

Motif analysis of ChIP-seq datasets for genome-wide mapping of ER $\alpha$  binding sites (Brown 2007, Stunnenberg 2009) revealed Oct-3/4 and Nkx3-1 binding sites to be enriched in the promoters of genes that recruit ER $\alpha$  preferentially or exclusively in the presence of tamoxifen and not estradiol (with >5 fold enrichment over estradiol) (Figure 5.1a). Thus, we validated ligand specific ER $\alpha$  recruitment to candidate genes (Figure 5.1b) by performing chromatin immunoprecipitation (ChIP) analysis and subsequently

confirmed the motif analysis by performing ChIP assays for Oct-3/4 demonstrating its specificity of recruitment upon tamoxifen but not estradiol treatment to the promoters of ER $\alpha$  target genes (Figure 5.1c). According to previous reports, MCF-7 cells express four isoforms of Oct-family of transcription factors namely, Oct-1, Oct-2, Oct-3/4 and Oct-11. We analyzed the expression of these transcripts in MCF-7 cells treated with estradiol or tamoxifen along a time-course of 4 and 24h and interestingly, found an up-regulation of Oct-3/4 and Oct-11 mRNA, but not Oct-1 or 2, specifically in the presence of tamoxifen (Figure 5.2a). By western blot analysis we also monitored protein levels for Oct factors upon tamoxifen treatment and found a significant increase only for Oct-3/4 (Figure 5.2b). To examine the effect of ER $\alpha$  depletion on tamoxifen driven Oct-3/4 induction, we used ICI (a known inhibitor of ER $\alpha$  function) as well as small interfering RNA (siRNA) mediated knockdown of ER $\alpha$  in the presence or absence of ligand (E2 and TOT), in MCF-7 breast cancer cells (Figure 5.2c). By both approaches we were able to reverse the tamoxifen specific induction of Oct-3/4 thereby highlighting the role of ER $\alpha$  in this mechanism.

### ***Nkx3-1 Is Required for Tamoxifen Mediated Induction of Oct-3/4 Gene***

Bioinformatic analysis for transcription factor binding sites enriched in gene promoters revealed an Nkx-binding site in the Oct-3/4 gene promoter (Figure 5.3a) and since this site was also found enriched in the promoters of targets of tam-bound ER $\alpha$ , we explored the possibility of involvement of Nkx3-1 in this tamoxifen driven induction of Oct-3/4. We then investigated the role of Nkx3-1 by performing small interfering RNA (siRNA) mediated knockdown of Nkx3-1 in the presence or absence of ligand (E2 and TOT), in MCF-7 breast cancer cells followed by quantitative real-time PCR or western blot analysis of expression of Oct-3/4 transcript and protein respectively. By siRNA directed against Nkx3-1 we reduced Nkx3-1 protein levels to ~15% of that in the scrambled siRNA treated cells and we observed a significant enhancement in tamoxifen mediated induction of Oct-3/4 both at the transcript (Figure 5.3b) and



protein level (Figure 5.3c). To establish the role of Nkx3-1 in tamoxifen driven Oct-3/4 induction through direct recruitment to its binding site on the Oct-3/4 promoter we performed luciferase assay in MCF-7 cells with WT or Nkx-mut Oct-3/4 promoter, with abrogated Nkx-binding site, fused to 2(ERE)-pS2-luciferase gene. We found a dose dependent decrease in tamoxifen induction of luciferase gene driven by WT-Oct-3/4 promoter upon Nkx3-1 over-expression, and a complete blockage of this reduction in the presence of Oct-3/4 promoter with mutated Nkx-binding site (Oct-3/4-Luc-mutNkx) and hence insensitive to Nkx3-1 (Figure 5.3d). To further confirm that Oct-3/4 gene is a primary target of Nkx3-1 we performed chromatin immunoprecipitation (ChIP) assay to examine the recruitment of Nkx3-1 to Oct-3/4 gene in the presence of estradiol or tamoxifen and found Nkx3-1 to be recruited at significantly lower levels in the presence of tamoxifen when compared to estradiol (Figure 5.3e) while the recruitment of ER $\alpha$  was significantly higher with tamoxifen when compared to estradiol (Figure 5.3f). To further investigate the mechanism of repression of Oct-3/4 promoter by Nkx3-1 we performed ChIP assay for RNAPolIII-phospho-Ser5 in cells perturbed with siRNA against Nkx3-1 and found a significant increase in active polymerase-II recruitment in cells with reduced Nkx3-1 protein (Figure 5.3g). Our results suggest that Nkx3-1 acts as a repressor for Oct-3/4 gene by blocking active RNA polII recruitment to its promoter and its loss upon tamoxifen treatment results in higher recruitment of active polymerase-II and hence de-repression of Oct-3/4 promoter.

### ***Skp2 Interacts With and Degrades Nkx3-1 Specifically In the Presence of Tamoxifen***

To investigate the role of Skp2 in Nkx3-1 mediated induction of Oct-3/4 in the presence of tamoxifen, we performed co-immunoprecipitation assay in Cos-1 cells by over-expressing Skp2(Flag) and/or Nkx3-1(Myc) in the presence or absence of ligand, estradiol or tamoxifen for 1, 4 or 24h and immunoprecipitating with Myc-antibody to pull-down Nkx3-1 followed by western blotting for Skp2. Interestingly, we found the interaction between the two proteins to be tamoxifen dependent (Figure

5.4a). To confirm these observations in an endogenous setting we performed adenovirus mediated over-expression of Skp2 followed by treatment with estradiol or tamoxifen for 45min or 4h and immunoprecipitation with a specific Skp2 antibody and subsequently western blotting for Nkx3-1. Interestingly, endogenous Nkx3-1 protein in MCF-7 cells also interacted with Skp2 in a tamoxifen dependent manner as there was a significantly higher amount of Nkx3-1 that could be pulled down with Skp2 in cells that were treated with tamoxifen (Figure 5.4b). Furthermore, we over-expressed Skp2 by infecting MCF-7 cells with its adenovirus followed by 24h treatment with vehicle, estradiol or tamoxifen and found a significant decrease in the absolute level of Nkx3-1 protein upon Skp2 over-expression, specifically in the presence of tamoxifen (Figure 5.4c). Since Skp2 is an E3-ubiquitin ligase by function we investigated whether this loss of Nkx3-1 protein occurred through ubiquitin mediated proteasomal degradation of Nkx3-1 by Skp2. To this end, we performed ubiquitination assay for Nkx3-1 by transfecting Cos-1 cells with Nkx3-1(Myc) alone or Nkx3-1(Myc)/Skp2 together in the presence of over-expressed HA-Ubiquitin and treated the cells with Vehicle (0.1% ethanol), E2 or TOT before they were harvested and processed for immunoprecipitation with specific antibody against Myc-tag to pull down Nkx3-1 along with factors in complex. Our results show a significant increase in ubiquitinated Nkx3-1 upon over-expression of Skp2 only in the presence of tamoxifen (Figure 5.4d), as a likely mechanism by which Skp2 mediates tamoxifen specific degradation of Nkx3-1.

#### ***p38MAPK Catalyzed Phosphorylation of Skp2 at Serine-64 is Required for Degradation of Nkx3-1 by Skp2***

After establishing the importance of Skp2 in tamoxifen driven proteasomal degradation of Nkx3-1 we wanted to further investigate the mechanism involved in this regulation and the participating factors upstream of Skp2. We performed Skp2 mediated Nkx3-1 ubiquitination in the presence of various kinase inhibitors and found an effect exclusively in the presence of a specific p38MAPK inhibitor (data not

shown). Bioinformatic analysis revealed Serine-64 residue in Skp2 as a putative p38MAPK target site which was later confirmed by both mass-spectrometry analysis for Skp2 in the presence of p38MAPK over-expression in MCF-7 cells (Figure 5.5b) and In-cell kinase assay for Skp2-WT or Ser-64A mutant in the presence of either p38MAPK-WT (wildtype) or p38MAPK-DN (dominant negative kinase dead mutant) over-expression in Cos-1 cells (Figure 5.5c). We next wanted to investigate if this phosphorylation of Serine-64 in Skp2 by p38MAPK is ligand dependent. In-cell kinase assay was performed in Cos-1 cells by over-expression of Skp2-WT or Skp2-S64A mutant with or without p38MAPK-WT, this time in the absence or presence of ligand, Estradiol (E2) or Tamoxifen (TOT). Interestingly, p38MAPK catalyzed phosphorylation of Skp2 at Ser-64 was observed specifically in the presence of tamoxifen (shown by the upward shift of Skp2 band) (Figure 5.5d). To address the possibility of the role of phosphorylation of Serine-64 in Skp2 mediated Nkx3-1 turnover, we performed ubiquitination assay for Nkx3-1 in the absence or presence of over-expressed Skp2-WT (wildtype Skp2), Skp2-S64A (Ser-64 phosphorylation defective mutant) or Skp2-S64E (constitutively active Ser-64 phospho-mimic) in Cos-1 cells and found a block in the interaction of phosphorylation defective Skp2-S64A mutant with Nkx3-1 and hence also in its ubiquitination by the ubiquitin ligase while the constitutively active Skp2-S64E mutant was able to efficiently interact with and ubiquitinate Nkx3-1, just like wildtype Skp2 thereby strengthening the idea of tamoxifen dependent Nkx3-1 turnover by Skp2, driven by tamoxifen dependent phosphorylation of Skp2 at Ser-64 by p38MAPK (Figure 5.7a). Lastly, to highlight the importance of regulation through Ser-64 site in Skp2 we performed sequence alignment of Skp2 peptide containing Serine-64 residue obtained from various species (as shown) and found a high degree of sequence conservation in the entire 15 amino acid long peptide containing Ser-64 residue in Skp2, across all species analyzed (Figure 5.7b).

***p38MAPK Initiated and Skp2 Mediated Nkx3-1 Degradation is Required for Tamoxifen Specific Induction of Oct-3/4 Gene***

Adenovirus mediated over-expression of either Skp2 or p38MAPK further confirmed the tamoxifen, not estradiol, driven induction of Oct-3/4 transcripts and protein in MCF-7 cells, as measured by quantitative real-time analysis (Figure 5.8a) and western blot analysis (Figure 5.8b) respectively thereby establishing a role for both Skp2 and p38MAPK in tamoxifen driven Oct-3/4 induction. Since Nkx3-1 had been previously shown to be required for tamoxifen driven induction of Oct-3/4, we investigated the role of Skp2 and p38MAPK in Nkx3-1 mediated Oct-3/4 regulation. To this end, we performed co-immunoprecipitation assay in MCF-7 cells treated with vehicle, E2 or TOT using a specific antibody against Nkx3-1 and found Skp2, p38MAPK and ER $\alpha$  in complex with Nkx3-1 specifically in the presence of tamoxifen (Figure 5.9a). We next investigated the role of p38MAPK initiated and Skp2 mediated tamoxifen driven degradation of Nkx3-1, previously shown to be directly recruited to Oct-3/4 promoter, in regulating induction of Oct-3/4 gene by tamoxifen. Chromatin immunoprecipitation (ChIP) assay in the presence of over-expressed Skp2 or p38MAPK followed by ligand treatment with estradiol or tamoxifen showed much greater recruitment of Nkx3-1 on Oct-3/4 promoter in the presence of estradiol compared to tamoxifen in control cells while in cells with over-expressed Skp2 or p38MAPK there was a dramatic reduction in the recruitment of Nkx3-1 in the presence of tamoxifen with no significant impact on untreated cells or cells treated with estradiol (Figure 5.8c and d). In the same experiment as described in Figure 4.8c, d the recruitment of ER $\alpha$ , which was appreciably higher with tamoxifen compared to estradiol in control cells, was unaffected by Skp2 or p38MAPK over-expression (data not shown). Upon subsequent investigation by ChIP for Nkx3-1 followed by reChIP for ER $\alpha$ , Skp2 or p38MAPK we found all these proteins to be in the same transcriptional complex on Oct-3/4 promoter

(Figure 5.9b) thereby suggesting the crosstalk between ER $\alpha$ , Skp2, p38MAPK and Nkx3-1 to be key for tamoxifen specific regulation of Oct-3/4.

## DISCUSSION

In this study we identified and characterized Oct-3/4, a member of the POU (Pou-Oct-Unc) superfamily of transcription factors, to be a co-regulator of ER $\alpha$  target gene expression rather preferentially in response to tamoxifen, not estradiol. Studies in embryonic stem (ES) cells have suggested Oct-3/4 as a critical and concentration dependent determinant of cell fate both in embryonic development and adult life, describing a central role for it in self-renewal, pluripotency, differentiation and lineage commitment (Pesce M et al., 2001; Tai M-H et al., 2005). Amongst many reports on the role of Oct-family of transcription factors in maintaining the pluripotency of ES-cells via their involvement in gene regulation upon direct recruitment to target gene promoters (Loh Y-H et al., 2006; Jin VX et al., 2007; Chakravarthy H et al., 2008; Sharov AA et al., 2008; Tantin D et al., 2008; Chavez L et al., 2009), very little is known in the field of breast cancer. Our first cue for the involvement of Oct-3/4 in ER $\alpha$  actions came upon our analysis of ChIP-seq datasets for mapping the genome-wide ER $\alpha$  binding sites, in MCF-7 breast cancer cells, in the presence of estradiol or tamoxifen (Stunnenberg 2009). In our computational motif analysis, we observed Oct-3/4 and Nkx3-1 transcription factor binding sites enriched in the promoters of target genes that recruit ER $\alpha$  exclusively or preferentially in the presence of tamoxifen when compared to estradiol, a phenomenon later validated by ChIP analysis of putative targets.

Further investigation revealed Oct-3/4 itself, but not other Oct-family isoforms such as Oct-1, 2 and 11 that are also expressed in MCF-7 breast cancer cells (Jin T et al., 1999), as one of the candidate genes that recruited ER $\alpha$  to its promoter preferentially in the presence of tamoxifen, not estradiol.

Consistent with this we observed an ER $\alpha$  dependent induction of Oct-3/4 transcript and protein in response to tamoxifen not estradiol. Notably, there was some induction also observed for Oct-11 transcript, not Oct-1 or 2, however, the effects failed to translate at the protein level. Although Oct-3/4 expression has been reported in various human cancers (Jin T et al., 1999; Monk M et al., 2001) and its role in tumorigenesis is well documented (Gidekel S et al., 2003; Hochedlinger K et al., 2005), the precise mechanisms by which its levels are regulated is not well understood. Recently it has been shown that the ubiquitous expression of Oct-3/4 in transgenic mouse embryos was associated with lethality (Ramos-Mejia V et al., 2005) as was its complete loss (Nichols J et al., 1998), thereby accentuating the need for its precise regulation. Our work presents a novel mechanism for the regulation of Oct-3/4 expression in the breast through its induction specifically by tamoxifen not estradiol bound ER $\alpha$ , via involvement of Nkx3-1, a transcription factor and a known repressor of ER $\alpha$  target gene expression (Holmes KA et al., 2008; Lupien M et al., 2009) that has been shown to inhibit ER $\alpha$  binding to genomic loci (Holmes K et al., 2008) and whose binding sites were found enriched in the Oct-3/4 promoter. Previous reports have identified genetic alterations leading to a reduction or a complete absence of Nkx3-1 expression in ~50% of prostate intraepithelial neoplasia (PIN) lesions and primary prostate tumors and in as many as ~80% of all metastatic tumors (Bowen et al., 2000) implicating a strong role for the transcription factor in inducing prostate tumorigenesis. Conversely, over-expression of Nkx3-1 inhibits cell proliferation and anchorage-independent growth in vitro, and suppresses tumor growth in nude mice in vivo (Kim et al., 2002).

While the role of Nkx3-1 in prostate tumorigenesis is well documented, we are only beginning to understand its functions in the breast. Preliminary work from our laboratory and others demonstrated an impairment of breast cancer cell growth by virtue of Nkx3-1 actions as a co-repressor to ER $\alpha$ , reinforcing a role for Nkx3-1 as a tumor suppressor gene with its loss suggested to be a major initiating event in breast tumorigenesis. Greater insights for the role of Nkx3-1 as a co-repressor of ER $\alpha$  actions

and hence its function as a tumor suppressor in the breast came following our recent work that highlights a critical role for Nkx3-1 in the repression of Oct-3/4 transcription by blocking the recruitment of RNA-Pol-II-phospho-Ser5 in addition to that of ER $\alpha$ , to the Oct-3/4 promoter, in both basal and estradiol treated cells. However, in the presence of tamoxifen, there was a loss of Nkx3-1 protein, later suggested to be due to its ubiquitin-proteasome mediated degradation by Skp2, an E-3 ubiquitin ligase and a major regulator of the G1/S cell cycle checkpoint and therefore cell cycle progression. Our findings are consistent with some other reports on the ubiquitin-proteasome dependent degradation of Nkx3-1 upon treatment with inflammatory cytokines, such as tumor necrosis factor  $\alpha$  and interleukin-1 $\beta$  (Markowski et al., 2008).

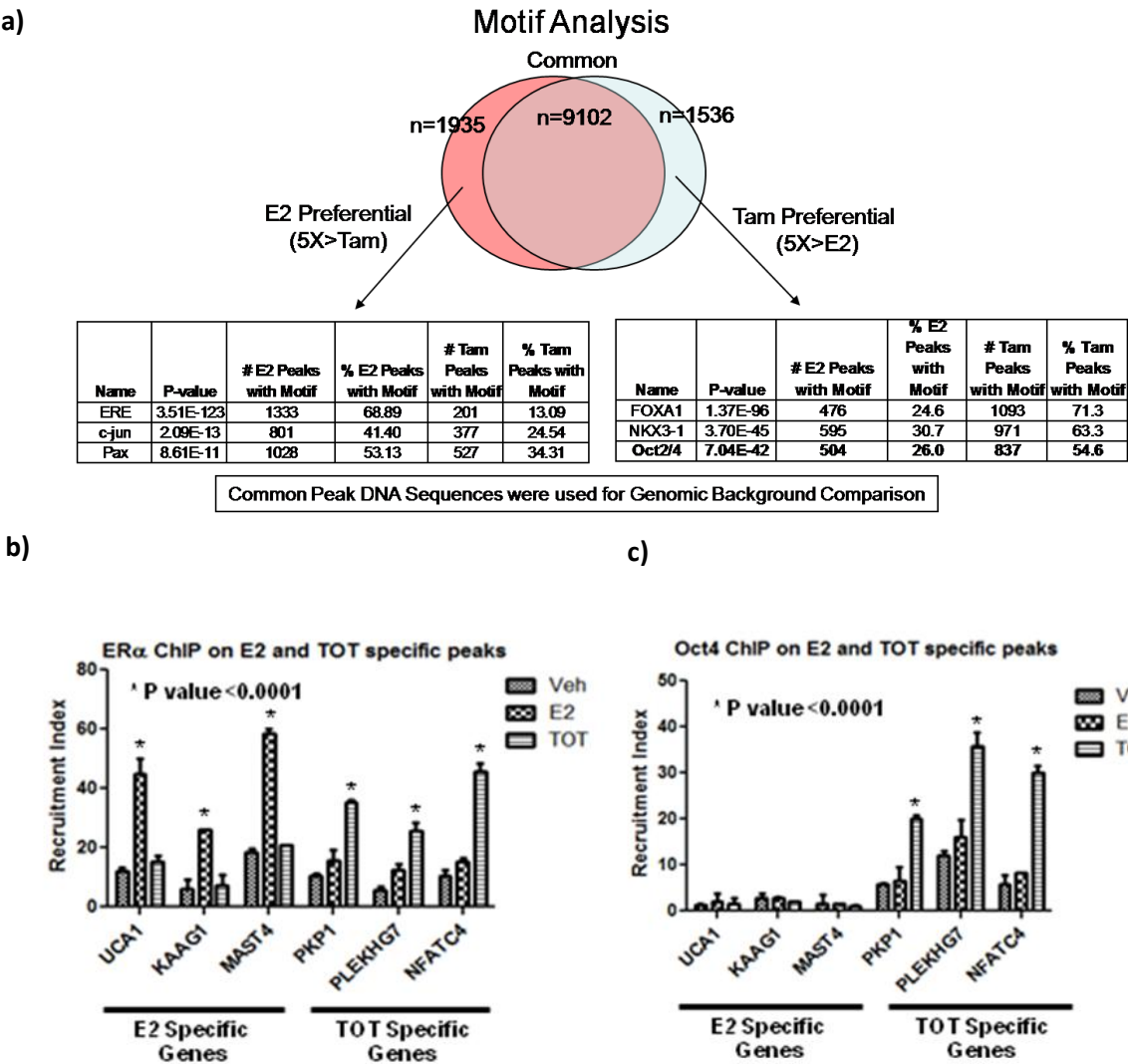
Interestingly, this tamoxifen induced Nkx3-1 degradation leading to the de-repression of the Oct-3/4 promoter required tamoxifen dependent phosphorylation of Skp2 at serine-64 residue by p38MAPK, known to be highly active in MCF-7-tam<sup>r</sup> cells compared to MCF-7 parentals with a role in driving tamoxifen resistance in these cells (Zhang CC et al., 2000; Dowsett et al., 2003; Gutierrez MC et al., 2005; Massarweh S et al., 2008). Upon further investigation we found elevated expression of Oct-3/4 while reduced Nkx3-1 in tamoxifen resistant MCF-7-tam<sup>r</sup> cells compared to parental cells, thereby lending support to our hypothesis of a major role of Skp2/p38MAPK/Nkx3-1 mediated Oct-3/4 expression in driving tamoxifen resistance in these cells. Consistent with this, perturbation of Oct-3/4 levels in MCF-7-tam<sup>r</sup> cells significantly impacted their sensitivity to tamoxifen. Our finding are in line with previous reports in mice that suggest Oct-3/4 expression to be confined to the pluripotent cells of the developing embryo while being absent from the differentiated somatic stem cells (Rosner et al., 1990; Scholer et al., 1990b; Yeom et al., 1996; Pesce et al., 1998) and characterize it as a major determinant of proliferative index of cells.

Consequently, it is tempting to speculate a critical role for Oct-3/4 in driving aggressiveness, migration and invasion of breast cancer cells in response to tamoxifen possibly through modulation of

genes that are targets of tamoxifen bound ER $\alpha$ , the precise mechanism for which, including the candidate genes, will need to be determined upon subsequent investigation. Taken together, our work documents a novel p38MAPK-Skp2-Nkx3-1 axis that impacts on gene regulation unique to tamoxifen bound ER $\alpha$  thereby regulating the cellular levels of Oct-3/4, a key regulator of growth, and driving tamoxifen resistance in breast cancer cells. Targeting of this pathway, thereby blocking tamoxifen specific induction of Oct-3/4, may have far reaching clinical significance for it could circumvent or even reverse the acquisition of tamoxifen resistance thereby making tam-resistant tumors amenable to endocrine therapy.

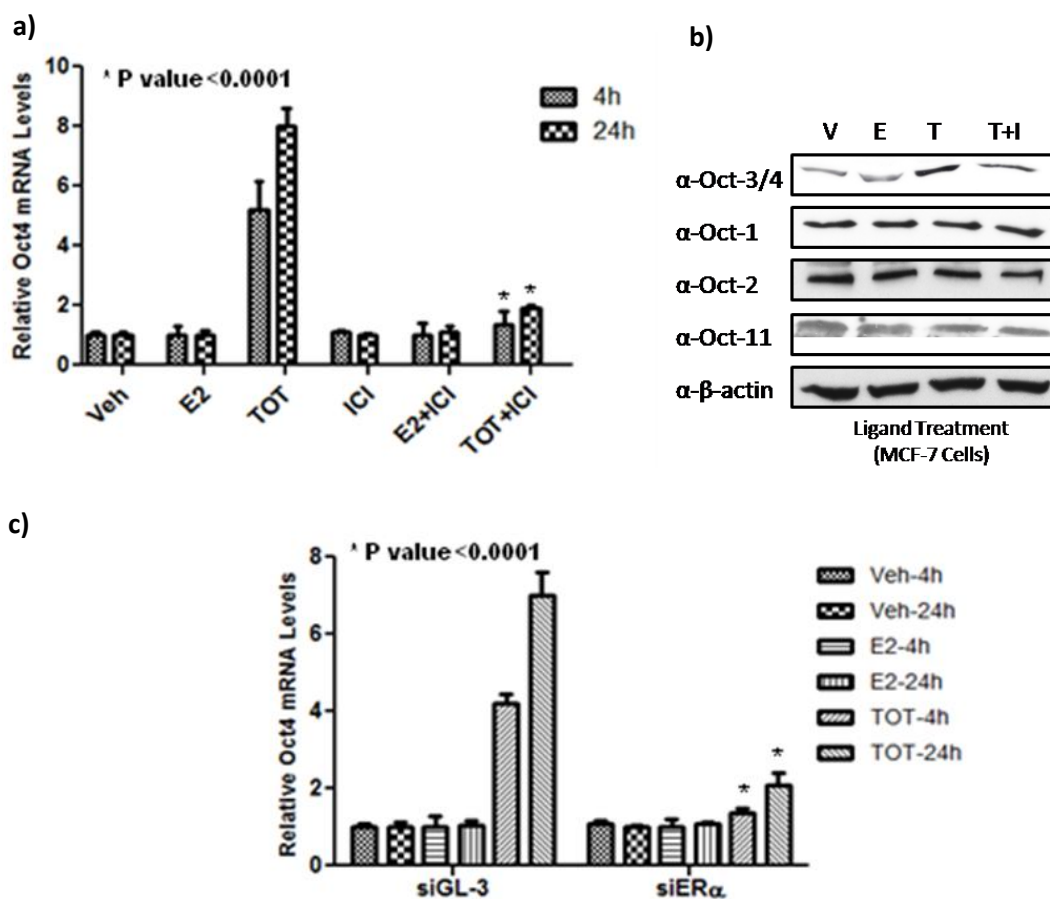


FIGURES



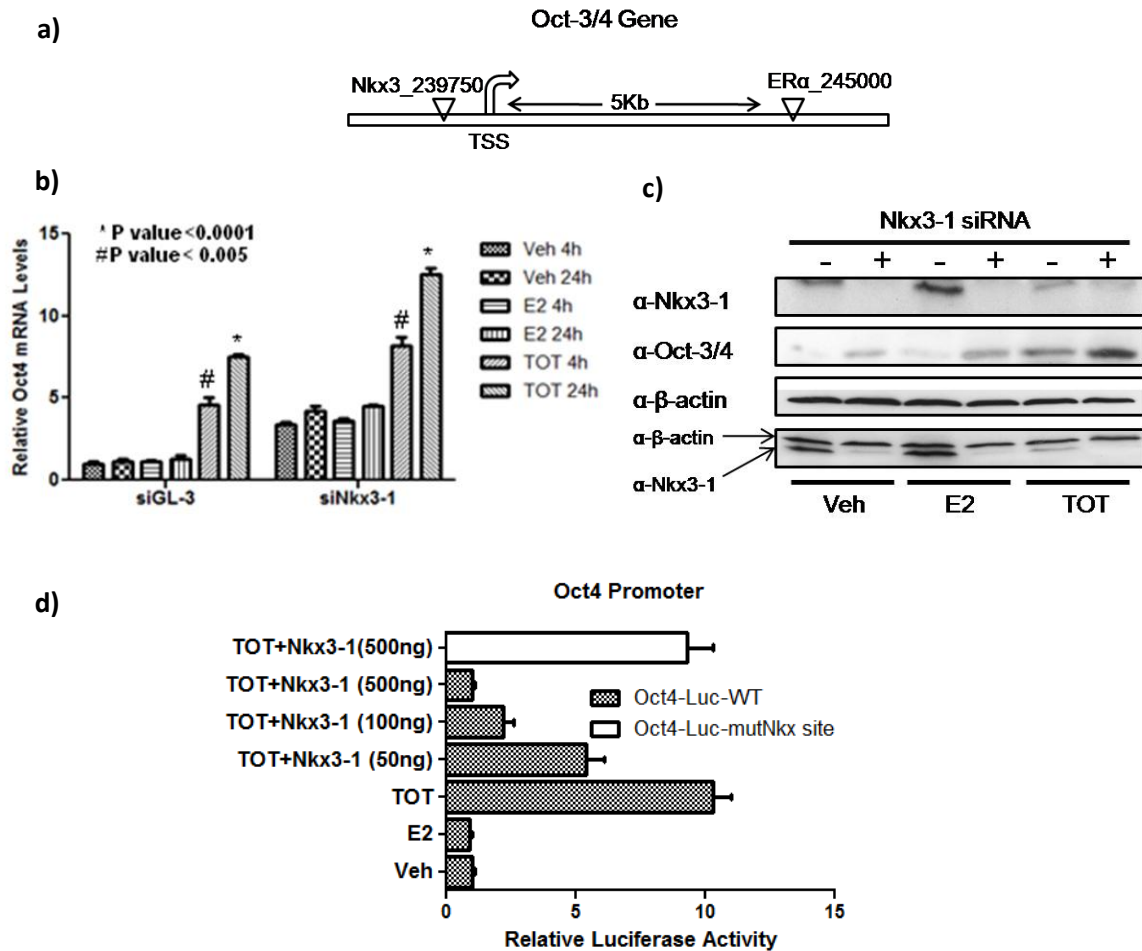
**Fig.5.1 Oct-3/4 is Binding sites are Enriched in the Promoters of Tam-bound ERα Target Genes**

a) Computational Motif Analysis: De novo motif analysis was performed as described in materials and methods. b) and c) Chromatin Immunoprecipitation (ChIP) assay: MCF-7 cells were treated with vehicle (0.1% ethanol), E2 (10nM) or TOT (1μM) for 24h and ChIP assays performed as described in materials and methods, using specific antibodies against ERα, Oct-4 or IgG negative control. Data are averages ± standard errors of the means of the results of three or more independent experiments and are represented as the recruitment index (specific antibody/IgG signal ratio).



**Fig.5.2 Oct-3/4 is Induced Specifically by Tamoxifen, not Estradiol In an ERα Dependent Manner**

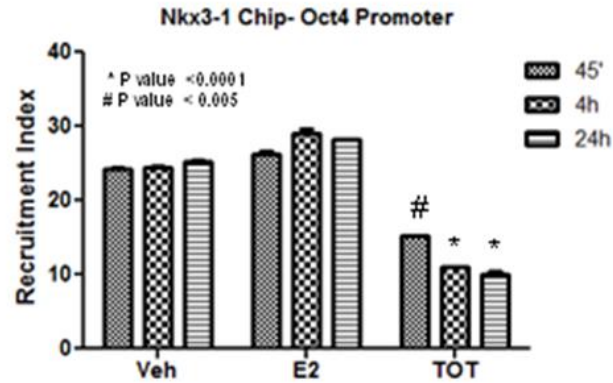
a) Quantitative real-time analysis: Q-PCR analysis to examine the expression of Oct-1, 2, 3/4 and 11, using 36B4 as internal control, in MCF-7 cells treated with vehicle (0.1% ethanol), E2 (10nM) or TOT (1μM), ICI (1μM), E2+ICI, TOT+ICI for 4 and 24h. b) Western blot analysis: Same as described in (a) with 24h samples processed for western blot analysis to examine Oct-3/4 protein. c) Quantitative real-time analysis: Q-PCR analysis to examine the expression of Oct-3/4 in MCF-7 cells transfected with either control non-targeting siRNA or siERα and subsequently treated with vehicle (0.1% ethanol), E2 (10nM) or TOT (1μM) for 4 and 24h.



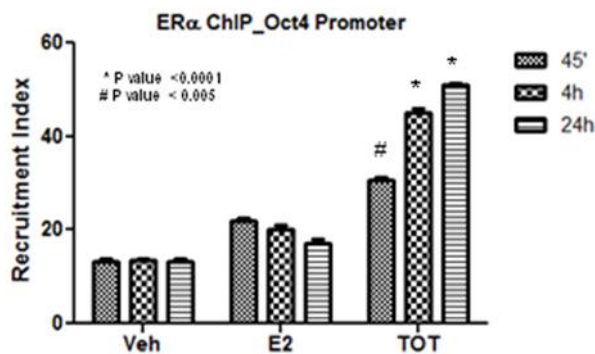
**Fig.5.3 Nkx3-1 is Required for Tamoxifen Mediated Induction of Oct-3/4 Gene**

a) Bioinformatics analysis: Transcription factor binding sites in Oct-3/4 promoter revealed a consensus site for Nkx3-1. b) Q-PCR analysis: Oct-3/4 expression in MCF-7 cells transfected with either control siRNA or siNkx3-1 and treated with vehicle, E2 (10nM) or TOT (1μM). c) Western blot analysis: Same as described in (b) with 24h ligand treatment samples processed to examine Oct-3/4 protein. d) Luciferase Reporter Assay: MCF-7 cells were transfected with pCMV vector and treated 6h post transfection with vehicle, E2 (10nM) or TOT (1μM) for 24h or transfected with increasing amounts of pCMV-Nkx3-1 and treated 6h post transfection with TOT (1μM) for 24h, in the presence of either Oct-3/4-Luc-WT (wildtype Oct-3/4 promoter) or Oct-3/4-Nkxmut-Luc (Oct-3/4 promoter with abrogated Nkx-binding site).

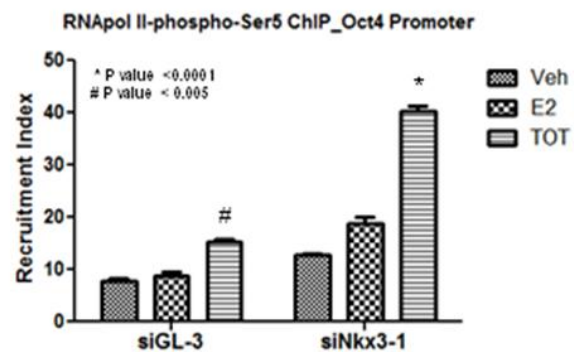
e)



f)

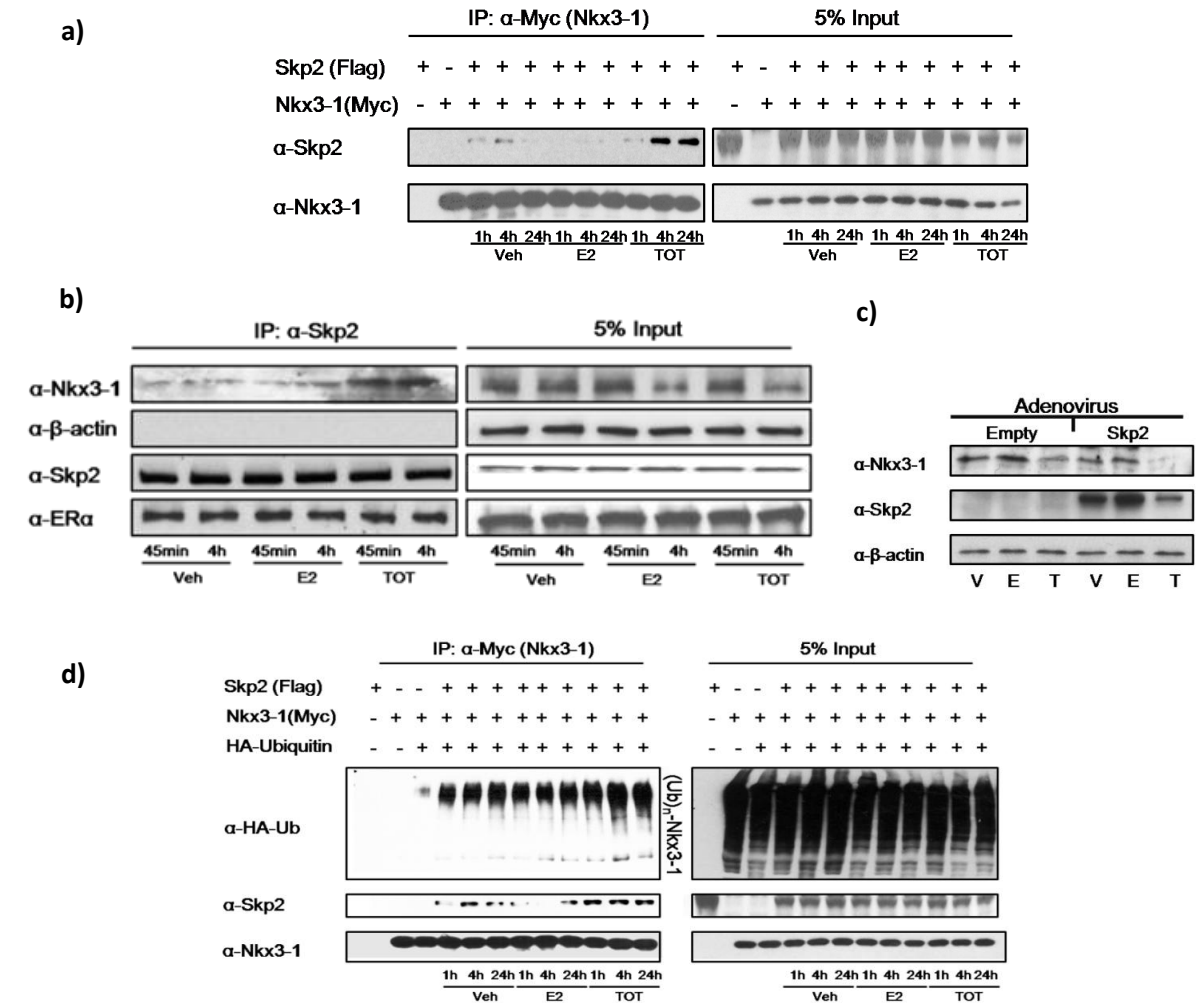


g)



**Fig.5.3 (contd.) Nkx3-1 is Required for Tamoxifen Mediated Induction of Oct-3/4 Gene**

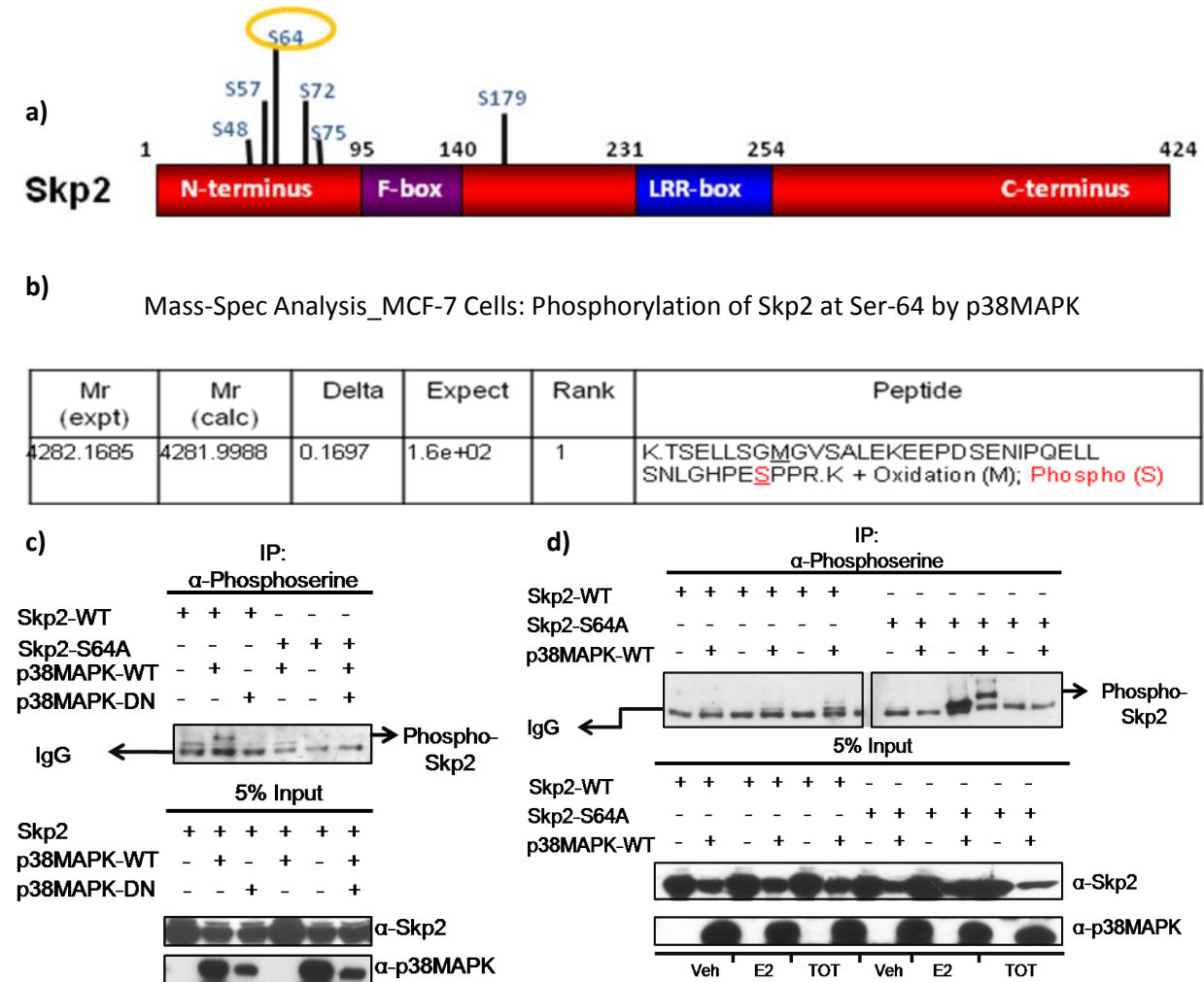
e) and f) Chromatin Immunoprecipitation (ChIP) assay: MCF-7 cells were treated with vehicle (0.1% ethanol), E2 (10nM) or TOT (1μM) for 45min, 4h and 24h and ChIP assays performed as described in materials and methods, using specific antibodies against Nkx3-1, ERα or IgG negative control. Data are averages ± standard errors of the means of the results of three or more independent experiments and are represented as the recruitment index (specific antibody/IgG signal ratio). g) ChIP assay for phospho-Serine 5-Pol II was performed in a parallel experiment as described in (e) and (f).



**Fig.5.4 Skp2 Interacts With and Degrades Nkx3-1 Preferentially In the Presence of Tamoxifen**

a) Cos-1 cells were transfected with either Myc-tagged Nkx3-1 or Flag-tagged Skp2 or both in the presence of vehicle, E2 (10nM) or TOT (1 $\mu$ M) for 1, 4 and 24h and subjected to immunoprecipitation using anti-Myc-tag antibody followed by western blotting with anti-Myc and anti-Skp2. b) Endogenous CoIP: MCF-7 cells were infected with Adeno-Skp2 and 24h post transfection treated with vehicle, E2 (10nM) or TOT (1 $\mu$ M) for 45min and 4h followed by immunoprecipitation using anti-Skp2 antibody and western blotting for Skp2 and Nkx3-1 and ER $\alpha$ . c) MCF-7 cells were infected with Adeno-Skp2 or control adenovirus ( $\beta$ -Gal virus) and 48h post transfection were treated with vehicle, E2 (10nM) or TOT (1 $\mu$ M)

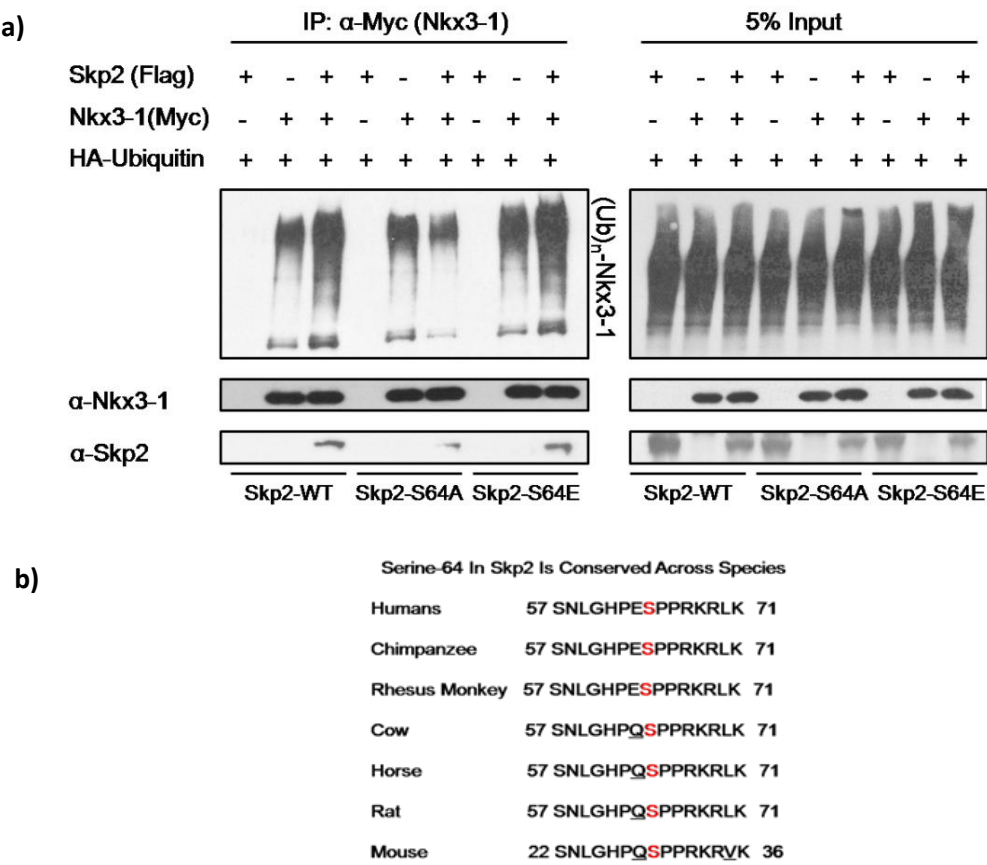
for 24h followed by western blotting for Nkx3-1. d) Ubiquitination Assay for Nkx3-1: Same as (a) also with HA-Ubiquitin (HA-Ub) expression and 5h MG-132 (10μM) treatment before harvest.



**Fig.5.5 p38MAPK Phosphorylates Skp2 at Serine-64 in a Tamoxifen Preferential Manner**

a) Schematic of Skp2 protein showing phosphorylation sites, esp. Serine-64. b) Mass-spec analysis: MCF-7 cells were infected with either adeno-Skp2(Flag) alone or with adeno-p38MAPK and harvested 48h after infection, subjected to immunoprecipitation using anti-Skp2 antibody followed by mass-spec analysis, as described before. c) In-Cell Kinase Assay: Cos-1 cells transfected with Skp2-WT or Ser-64A mutant with either p38MAPK-WT or p38MAPK-DN were subjected to immunoprecipitation using anti-

phosphoserine followed by WB with anti-Skp2 and p38MAPK antibody. d) In-cell kinase assay: Cos-1 cells transfected with Skp2-WT or Ser-64A in the presence of p38MAPK-WT were treated with Veh, E2 (10nM) or TOT (1μM) for 24h, 6h post transfection subjected to immunoprecipitation using anti-phosphoserine followed by WB with anti-Skp2 and p38MAPK antibody.

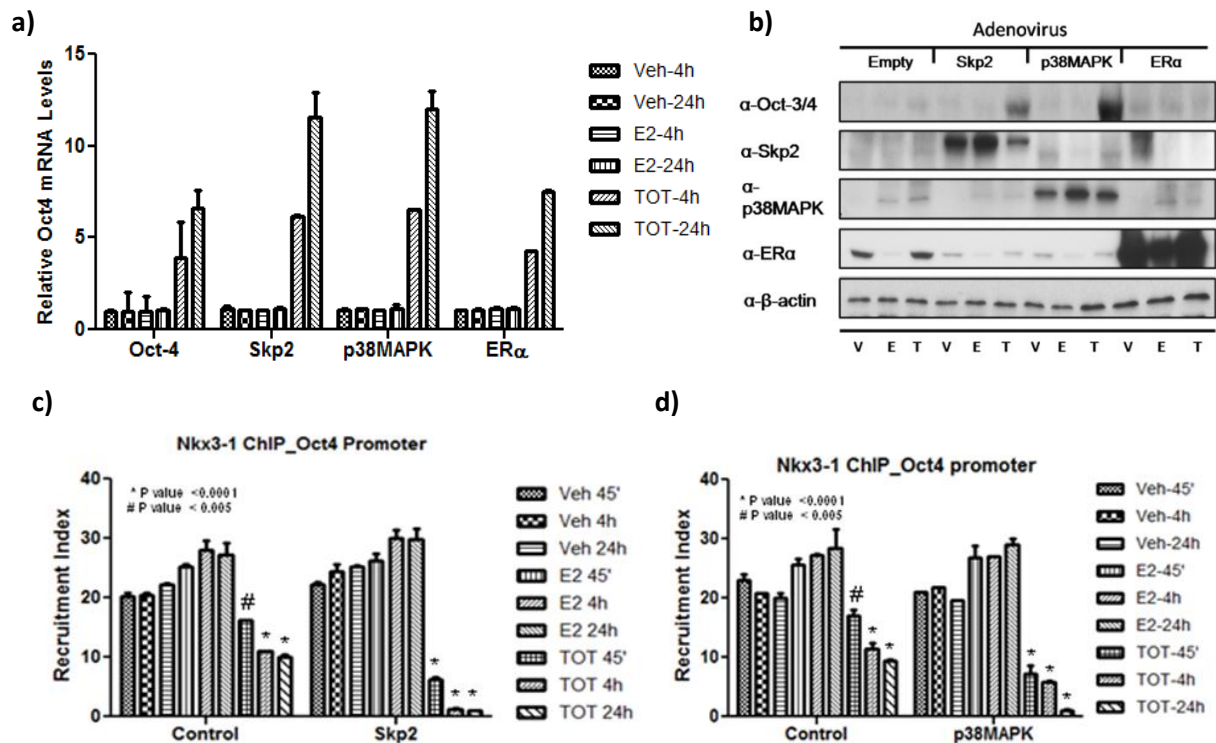


**Fig.5.6 p38MAPK Mediated Phosphorylation of Skp2 at Serine-64 Is Required for Nkx3-1 Degradation**

a) Ubiquitination Assay for Nkx3-1: Cos-1 cells were transfected with Skp2-WT, Skp2S64A or Skp2S64E along with Nkx3-1(Myc) and HA-Ubiquitin (HA-Ub) or alone, as indicated. Cells were treated with MG-132 (10μM) for 5h before harvest, 24h after transfection. Cell lysates were thereafter immunoprecipitated using anti-myc tag antibody to pull-down Nkx3-1 followed by western blotting with anti-Nkx3-1, anti-Skp2 and anti-HA-tag antibody to detect ubiquitinated Nkx3-1. b) Protein Sequence



Alignment: Alignment of Serine-64 containing peptide sequence of Skp2 protein obtained from various indicated species showing high degree of sequence conservation.



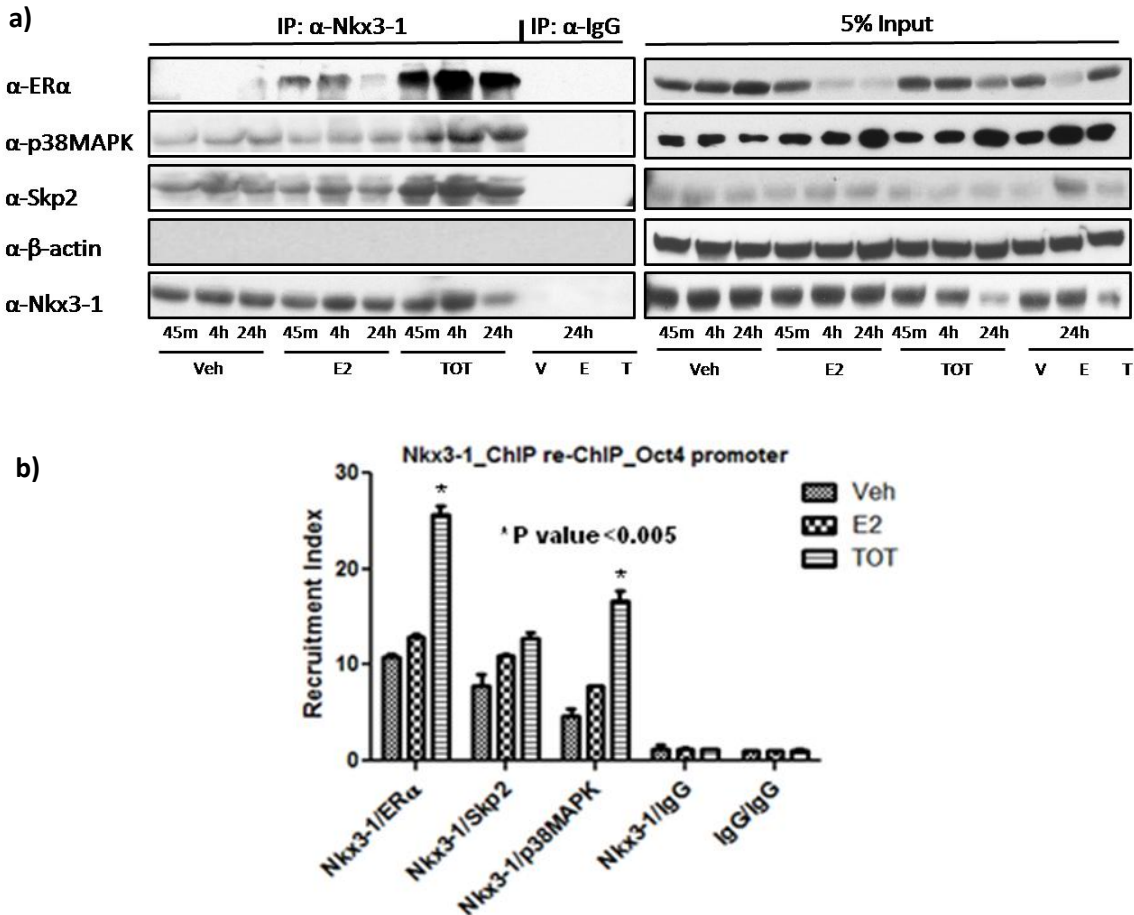
**Fig.5.7 p38MAPK Initiated and Skp2 Mediated Nkx3-1 Degradation is Required for Tamoxifen Specific Induction of Oct-3/4**

a) Quantitative real-time analysis: Q-PCR analysis to examine the expression of Oct-3/4 in MCF-7 cells infected with Adeno-Control ( $\beta$ -Gal), Adeno-Skp2 or Adeno-p38MAPK for 48h including 4 and 24h treatment with vehicle, E2 (10nM) or TOT (1 $\mu$ M), before harvest. 36B4 was used as an internal control.

b) Western blot analysis: Same experiment as described in (a) with 24h ligand treatment was processed for western blot analysis to examine Oct-3/4 protein.

c) and d) ChIP Assay for Nkx3-1: MCF-7 cells infected with Adeno-Control ( $\beta$ -Gal), Adeno-Skp2 or Adeno-p38MAPK for 48h followed by treatment with vehicle, E2 (10nM) or TOT (1 $\mu$ M) for 45min, 4h and 24h were subjected to ChIP assays performed as described in materials and methods, using specific antibodies against Nkx3-1 or IgG negative control.

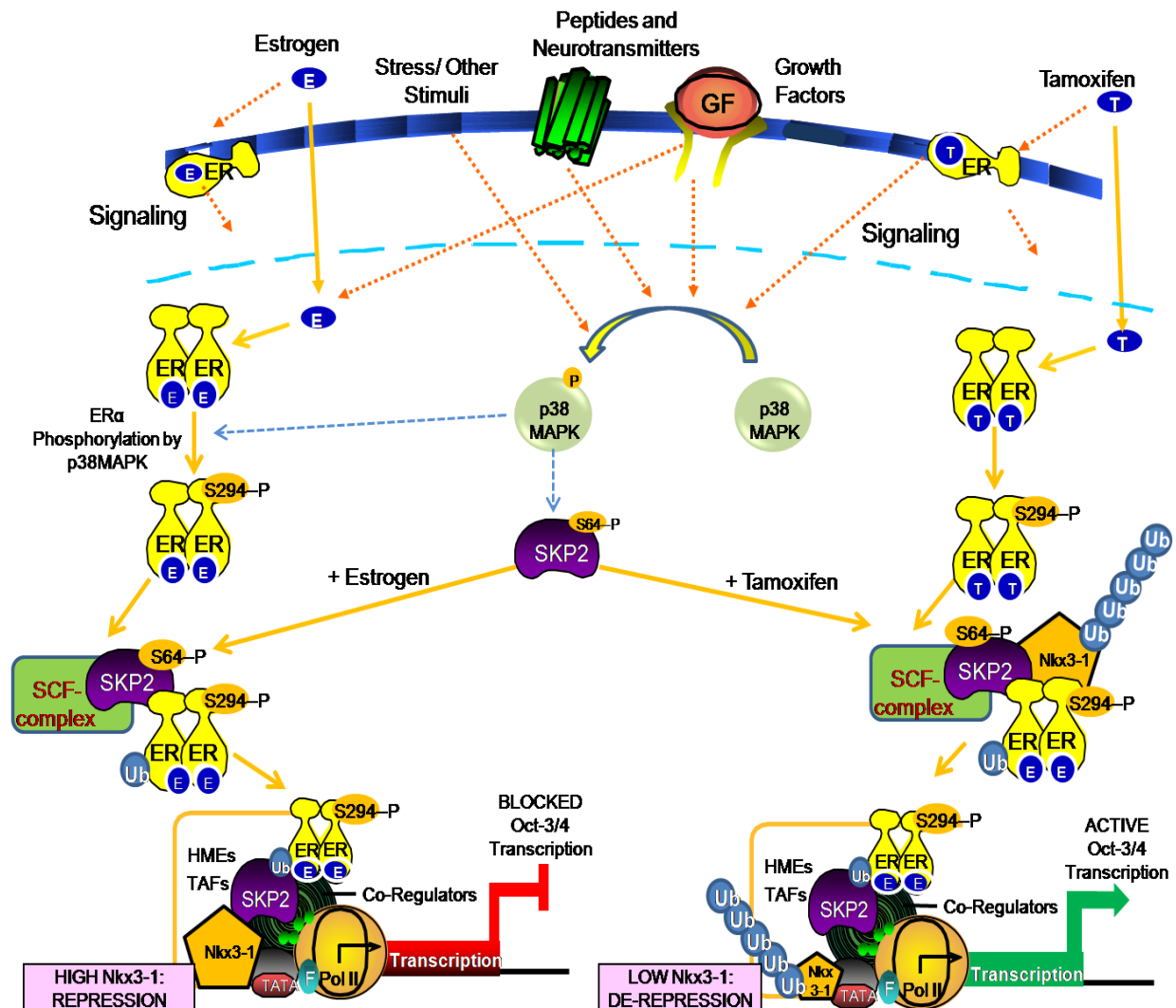




**Fig.5.8 p38MAPK and Skp2 Complex with Nkx3-1 to form part of ERα Transcriptional Complex on Oct-3/4 Promoter**

a) Co-immunoprecipitation (CoIP) assay: MCF-7 cells were treated with vehicle, estradiol or tamoxifen for 45min, 4h and 24h and thereafter subjected to immunoprecipitation using anti-Nkx3-1 antibody to pull-down Nkx3-1 followed by western blotting with anti-Nkx3-1, anti-ERα, anti-p38MAPK and anti-Skp2 antibodies. b) ChIP re-ChIP: MCF-7 cells treated with vehicle (0.1% ethanol), E2 (10nM) or TOT (1μM) for 4h were subjected to ChIP assays performed as described in materials and methods, using specific antibodies against Nkx3-1 or IgG negative control. After the first pull-down, immunoprecipitated material was recovered with 10mM dithiothreitol in IP buffer at 37°C for 30min, diluted and subjected to

a second round of immunoprecipitation using specific ER $\alpha$ , Skp2, p38MAPK antibodies or IgG negative control.

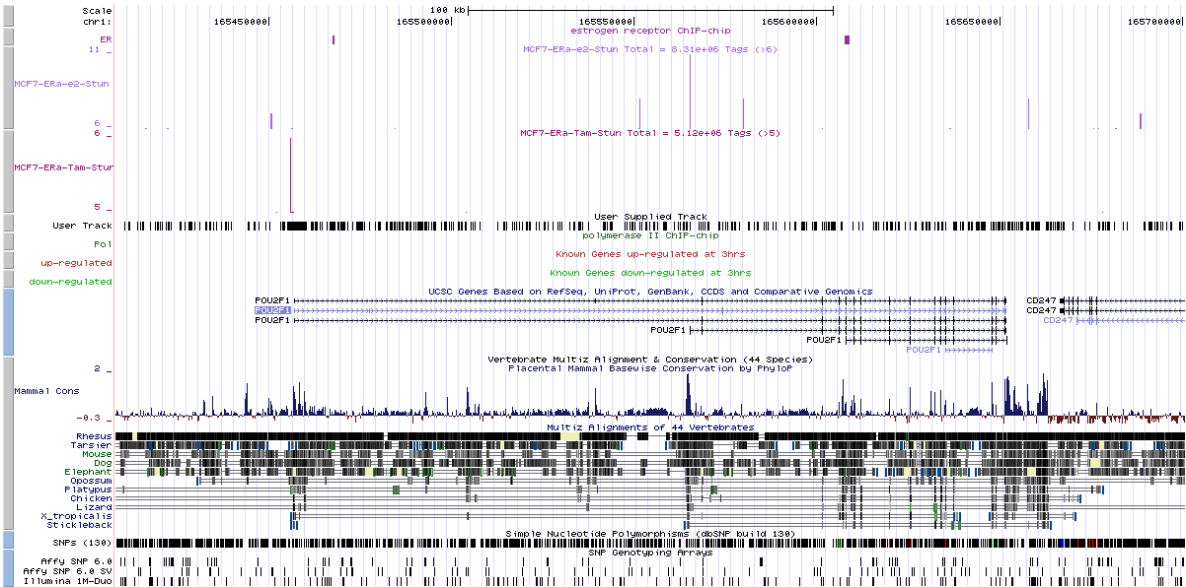


**Fig.5.9 MODEL**

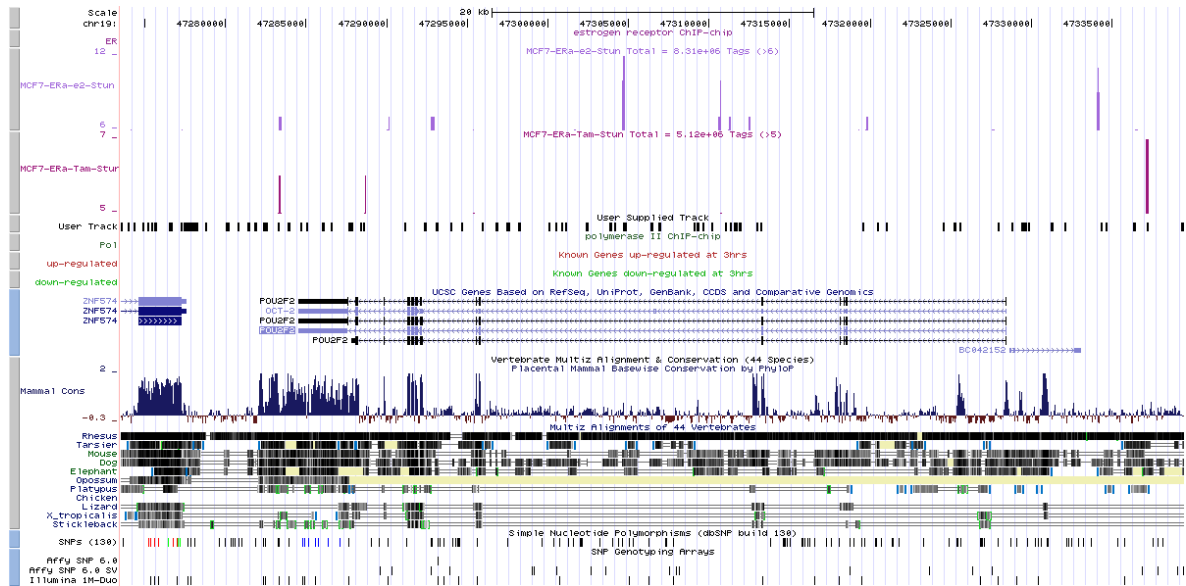
Schematic model highlighting the tamoxifen driven and p38MAPK catalyzed phosphorylation of Skp2 at Serine-64 drives ubiquitin dependent proteasomal degradation of Nkx3-1 exclusively in the presence of tamoxifen thereby inducing Oct-3/4 gene only in the presence of tamoxifen, not estradiol in breast cancer cells.

SUPPLEMENTARY FIGURES

ERα Recruitment to Oct-1 Promoter in the Presence of E2 (purple) or Tamoxifen (pink)



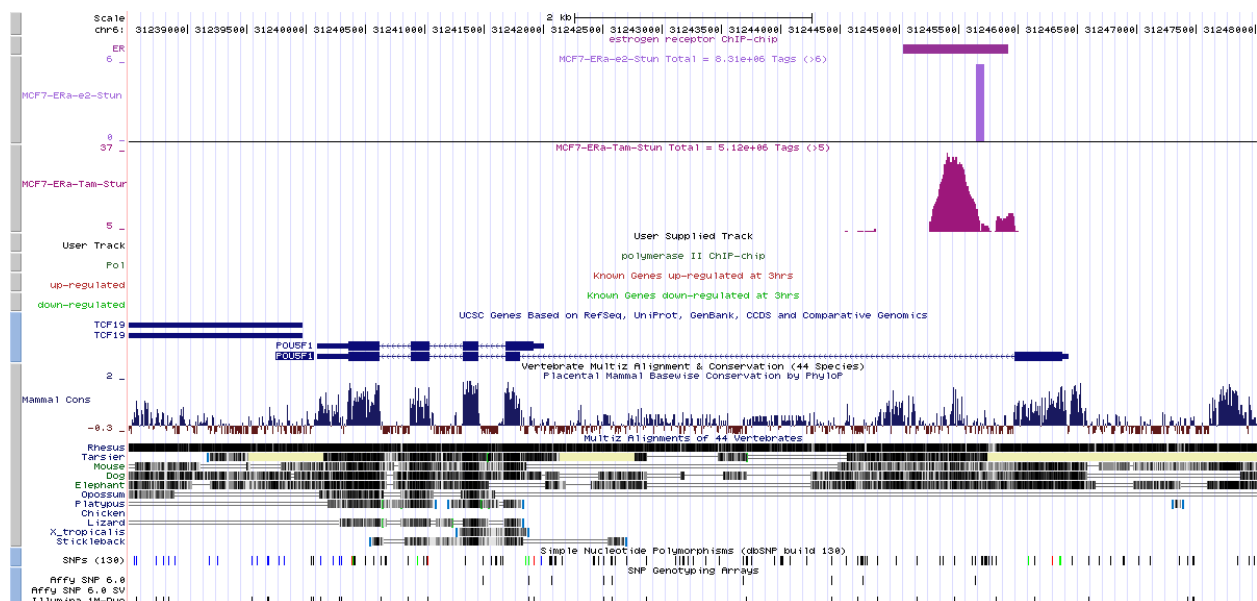
ERα Recruitment to Oct-2 Promoter in the Presence of E2 (purple) or Tamoxifen (pink)



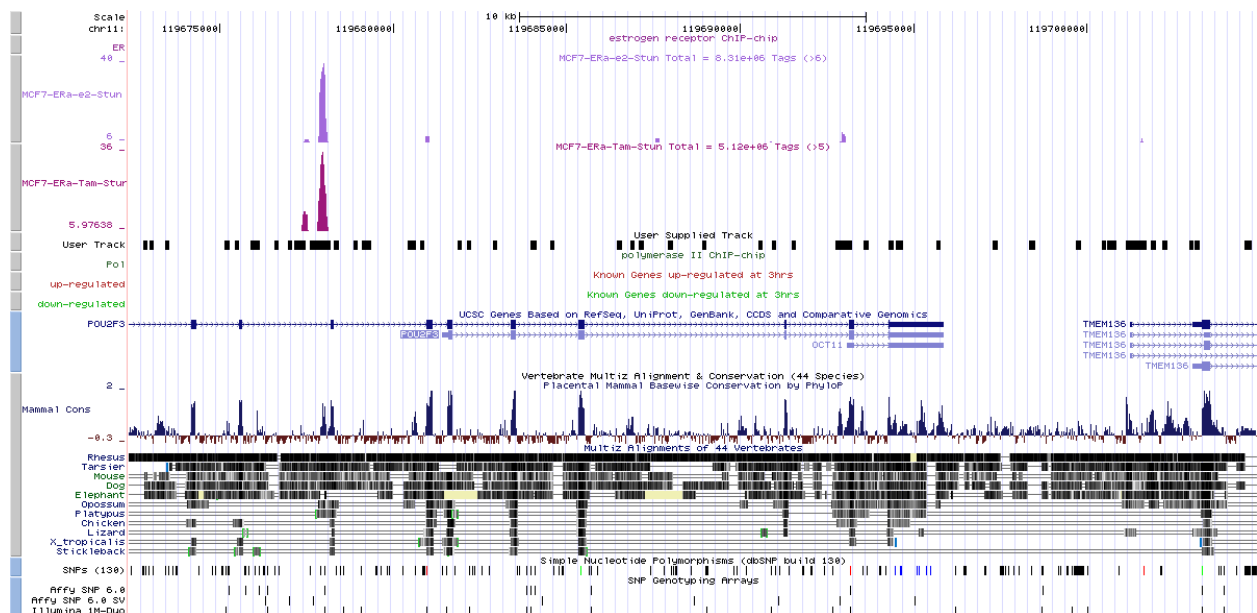
S1. UCSC Genome Browser snapshots for the Recruitment of ERα to Oct Gene Promoter(s)

For Oct-1 and Oct-2 gene promoters

## ERα Recruitment to Oct-3/4 Promoter in the Presence of E2 (purple) or Tamoxifen (pink)



## ERα Recruitment to Oct-11 Promoter in the Presence of E2 (purple) or Tamoxifen (pink)



### S1.contd. UCSC Genome Browser snapshots for the Recruitment of ERα to Oct Gene Promoter(s)

For Oct-3/4 and Oct-11 gene promoters

## ACKNOWLEDGEMENTS

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## **CHAPTER VI- ROLE OF SKP2 IN REGULATING TAMOXIFEN SENSITIVITY OF BREAST CANCER CELLS VIA OCT-3/4-ER $\alpha$ TARGET GENES**

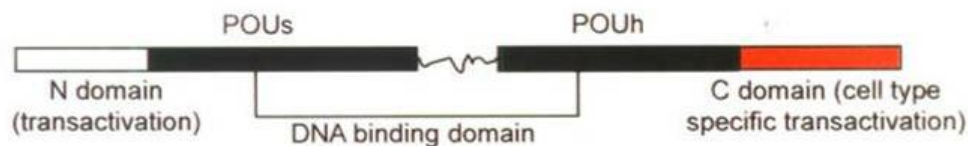
### **ABSTRACT**

After elucidating the mechanism for its tamoxifen specific induction in breast cancer cells, we next established the role of Oct-3/4 in regulating ER $\alpha$  target gene expression and acquisition of tamoxifen resistance by these cells. We found NFATC4 as an ER $\alpha$  target induced preferentially in the presence of tamoxifen, both at the transcript and protein level, and co-regulated by Oct-3/4, through specific Oct-response elements in its promoter. Interestingly, Oct-3/4 was found in complex with ER $\alpha$  specifically in the presence of tamoxifen, not estradiol and binding of Oct to the NFATC4 gene promoter appeared essential for ER $\alpha$  recruitment to ER response elements in close proximity. Also further investigation demonstrated Skp2 and p38MAPK to impact positively on ER $\alpha$ -Oct-3/4 driven NFATC4 gene expression upon tamoxifen treatment. This was largely due to Skp2 and p38MAPK recruitment to the NFATC4 gene promoter as part of the Oct-3/4-ER $\alpha$  transcriptional complex, in a tamoxifen dependent manner. Growth assays in MCF-7-tamr cells, with modulated levels of Oct-3/4, with either siRNA mediated knockdown or adenovirus mediated over-expression in the presence of tamoxifen, established a critical role for Oct-3/4 in acquired tamoxifen resistance and its elevated levels in these tamoxifen resistant derivatives compared to parental MCF-7 cells, further supported the hypothesis. Finally, NFATC4 was found to increase the activation of p38MAPK, a factor with established role for driving tamoxifen resistance in these cells, in a tamoxifen dependent manner through a positive feedback mechanism. Our work therefore, highlights a novel mechanism for tamoxifen specific induction of Oct-3/4 and its subsequent role in regulating tamoxifen bound ER $\alpha$  target genes through involvement of p38MAPK and Skp2 thereby controlling tamoxifen sensitivity of breast cancer cells.



## INTRODUCTION

Embryonic development in mammals is controlled by regulatory genes, some of which regulate the transcription of other genes. These regulators activate or repress patterns of gene expression that mediate phenotypic changes in cells during differentiation. Oct4 (also known as Oct-3), which belongs to the POU (Pit-Oct-Unc) transcription factor family, is one such key regulator of embryonic development. The POU family of transcription factors can activate the expression of their target genes through binding an octameric sequence motif of an AGTCAAAT consensus sequence.



Pan G.J et al., Cell Research (2002); 12(5-6):321-329

### ***Fig.6.1 Structure of Oct-4, a POU-family of transcription factor***

Structure and Function of Oct4 A. a schematic illustration of Oct4 domains. Note the C-domain behaves differently from the N-domain with respect to cell type-specific transactivation.

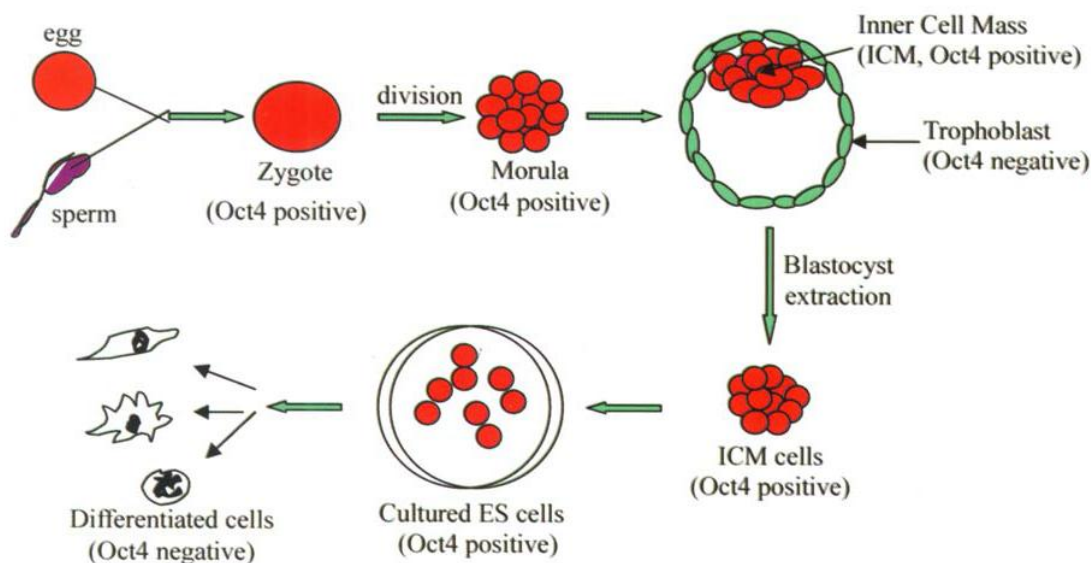
The hallmark feature of the POU family of transcription factors is the POU domain, which consists of two structurally independent subdomains: a 75 amino acid amino-terminal POU specific (POUs) region and a 60 amino-acid carboxyl-terminal homeodomain (POUh). Both domains make specific contact with DNA through a helix-turn helix structure and are connected by a variable linker of 15 to 56 amino-acids. Regions outside the POU domain are not critical for DNA binding and exhibit little sequence conservation. The N-terminal domain (N domain) is rich in proline and acidic residues, while the C-terminal domain (C domain) is rich in proline, serine and threonine residues. The N domain has traditionally been accepted for its role in transactivation. More recent data suggest that the C domain also plays a role in transactivation. It was subsequently demonstrated that the activity of Oct4 C domain

is cell type specific and is regulated through phosphorylation, whereas the N domain is not. The cell type specificity was observed only if the C domain was linked to the POU domains of Oct4 and Oct- 2, but not to Pit-1 or the Gal4 DNA binding domain. This finding suggests that Oct4 POU-domain may function differently by serving as interaction sites for cell type-specific regulatory factors. Since the cell-type-specific activity of regulatory factors ensures the expression of target genes in an orderly fashion during development, Oct4 and its functional partners may be regulated in a specific manner throughout mammalian embryogenesis. Indeed, Oct4 is expressed by germ cells from the totipotent zygote to the highly specialized oocyte. It is likely that Oct4 may function in concert with other regulators to activate specific target genes in specific cell types at defined developmental stages. The fact that the N domain differs from the C domain in activity and cell type specificity may help explain the functional diversity for Oct4. Furthermore, the C domain may activate certain targets, which do not respond to the N domain during development. Examination of Oct4 structure with respect to its diverse biological function is only the starting point in unraveling the regulatory circuits responsible for maintaining stem cell pluripotency and controlling the differentiation of stem cells to various cell types. Certainly, more detailed work will be required to understand the structural basis of Oct4 functionality.

### ***Regulation of Oct4 expression***

Given its critical role in maintaining pluripotency, Oct4 activity must be tightly regulated to ensure the continuity of the germline and proper differentiation of various tissues and organs. In the mouse, Oct4 mRNA is present in mature oocytes. At the eight cell stage, Oct4 expression reaches a much higher level. Subsequently, Oct4 expression becomes restricted to the ICM, and is down-regulated in the TE and primitive endoderm. Later on in embryo development, Oct4 expression is only maintained in primordial germ cells (PGCs). Hansis et al examined Oct4 expression in human blastocysts. The Inner Cell Mass

(ICM) and Trophoectoderm (TE) of 17 human blastocysts were separated and Oct4 mRNA level was individually assessed by RT-PCR. The results demonstrated that the mean Oct4 expression was 30 times higher in totipotent ICM cells than in differentiated TE cells. These studies suggest that the expression pattern of Oct4 is very similar between mouse and human cells [1, 2].



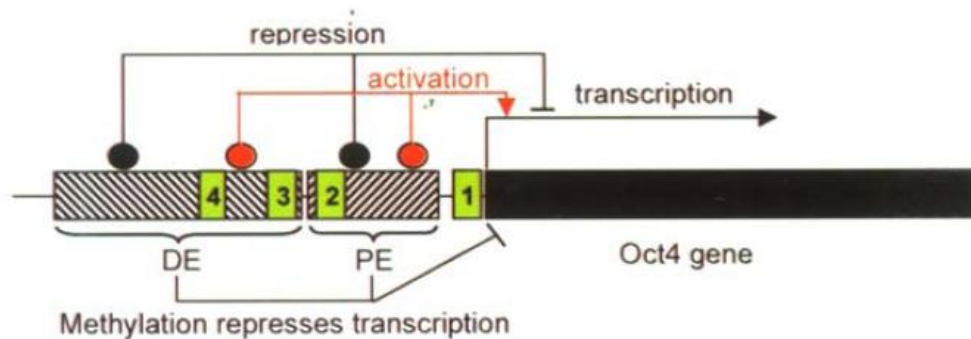
Courtesy: Pan G.J et al., Cell Research (2002); 12(5-6):321-329

### **Fig.6.2 ES cells and Oct4 expression**

The isolation and differentiation of ES cells in vitro are illustrated schematically starting with the fertilization of an egg by a sperm to form a zygote. At the blastocyst stage, inner cell mass (ICM) becomes visible and can be extracted and cultured in vitro to form embryonic stem (ES) cells. Cultured ES cells can be induced to differentiate into various cell types that are negative for Oct4. The stages of Oct4 expression are noted and the cells with Oct4 expression are marked in red colour. There is a general correlation between Oct4 expression and totipotency

Expression of Oct4 is regulated at the transcription level by cis-acting elements located upstream of the Oct4 gene and methylation of chromatin structure. By analyzing the expression of the LacZ reporter gene under the control of a 18Kb fragment from Oct4 genomic locus, Yeom et al identified two elements, which they named proximal enhancer (PE) and distal enhancer (DE) that may regulate the cell

-type-specific expression of Oct4. By using in vivo footprinting, they identified the precise binding sites for transcription factors within these two enhancers. One site, named 1A, was identified within the PE, and another site, named 2A, within the DE. Both sites exhibit nearly identical sequence homologous to the GC box and are crucial for the activity of PE and DE, respectively. But there was no further evidence to demonstrate the involvement of these two enhancers in stem cell specific activities in vivo.



Courtesy: Pan G.J et al., Cell Research (2002); 12(5-6):321-329

### ***Fig.6.3 Upstream regulatory elements of the Oct4 gene***

DE, distal enhancer, and PE, proximal enhancer, are important for regulating Oct4 expression. There are 4 regions that are highly conserved among human, bovine and mouse Oct4 promoter/enhancer elements, shown as green box 1 through 4 relative to DE and PE. Conserved region 1 (CR1) is downstream of PE and immediately upstream of exon 1. Each enhancer contains multiple potential binding sites for transcription factors that can either activate (red) or repress Oct4 expression. In addition, methylation in these regions represses Oct4 expression in differentiated cells.

On the other hand, Nordhoff et al comparatively analyzed the human, bovine, and murine Oct4 upstream promoter sequences and revealed four conserved regions of homology (CR1 to CR4) between these species (66-94% conservation). They found that element 1A in PE is located approximately half way between CR2 and CR3. A putative Sp1/Sp3 binding site and an overlapping hormone responsive element (HRE) in CR1 were found to be identical in all three species. In addition, there were a large number of CCC(A/T)CCC motifs, which exhibit various levels of homology within the upstream regions.

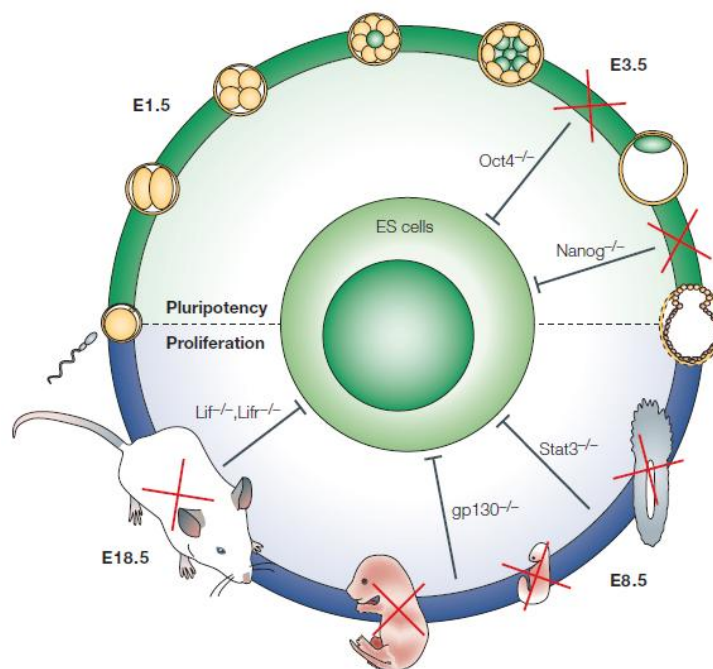
These sequences may be essential for Oct4 expression, thus further experimental investigation is necessary [3-7]. In addition, Hummelke and Cooney reported that germ cell nuclear factor (GCNF), an orphan nuclear receptor, could repress Oct4 gene activity by specially binding to the sequence within the PE. In agreement to this, GCNF expression inversely correlates with Oct4 expression in differentiating embryonic cells. In mouse embryos deficient in GCNF, the expression of Oct4 is no longer limited within the germ cell lineage after gastrulation, suggesting that GCNF is responsible for the repression of Oct4 gene expression during stem cell differentiation [8]. Further dissection of the Oct4 gene promoter/enhancers will reveal the precise cis-acting elements that bind to corresponding trans-acting factors, which act in concert to govern the lineage-specific expression or repression of Oct4. In addition to the cis-acting elements, there are additional mechanisms that may regulate the activity of Oct4.

One hypothesis is that the steady state level of Oct4 in totipotent cells may be a consequence of the establishment of active chromatin rather than the function of transcription activators. Ben-Shushan et al reported that the extinction of Oct4 activity in stem cells-fibroblast hybrid cells was accompanied by rapid methylation of regulatory sequences such as PE and DE in the Oct4 promoter/enhancer region. Jaenisch et al suggested that there must be a wave of de novo methylation occurring in the somatic cells of the embryo. These studies are consistent with the idea that methylation of the Oct4 regulatory sequences such as PE and DE shuts down Oct4 expression. PGCs, arising from extra-embryonic mesoderm and maintaining a steady level of Oct4 expression, may have a mechanism to prevent the methylation of their genome, at least within the Oct4 regulatory sequence. On the other hand, the maintenance of Oct4 expression in PGCs and oocytes may be due to the escape of chromatin from general reprogramming by methylation that occurs in epiblast cells at the time of gastrulation. It remains to be determined how ES cells integrate regulation by transcription factors and DNA methylation to either retain pluripotency or undergo differentiation into various cell types.

### ***The function of Oct4 in development and pluripotency***

In an effort to define the relationship between Oct4 expression and stem cell pluripotency, Niwa et al measured the levels of Oct4 expression at various ES cell states. These results indicated that Oct4 controls the pluripotency of stem cells in a quantitative fashion. Specifically, they determined that high level of Oct4 expression drives ES cells to endoderm and mesoderm lineages, while stem cells with low level of Oct4 differentiate into TE. Only a normal level of Oct4 can retain stem cells in a pluripotent state. These observations suggest that Oct4 is different from many known transcription regulators that appear to function in a binary on-off manner. In some cases, Oct4 can act as a repressor of target genes whereas in other cases, it acts as an activator. For example, Oct4 motifs were reported within the proximal promoters of  $\alpha$  and  $\beta$  subunit of human chorionic gonadotropin (hCG) genes. Oct4 serves as a repressor of both of these genes through binding to the octamer motifs in stem cells [9, 10]. In differentiated TE, Oct4 is down-regulated and no longer able to trans-repress hCG expression, signaling the reversal of a newly established gene expression pattern in these cells. Oct4 may function through other transcription factors to activate or repress target genes [11-15]. Members of the Forkhead Box (Fox) family have a winged-helix DNA binding structure and are strongly implicated in early embryonic lineage decisions, especially in the development of the endoderm and subsequent endodermal organogenesis. FoxD3, a member of this family, could bind to and activate the promoters of other members of this family, e.g., FoxA1 and FoxA2, while FoxA1 and FoxA2 are critical for the embryonic development of endodermal foregut organs. Guo et al reported that Oct4 could repress the expression of FoxA1 and FoxA2 through an interaction with the DNA binding domain of their activator FoxD3. Since Oct4 does not bind to the promoters of FoxA1 and FoxA2, it behaves as a co-repressor of these promoters. This report suggests that Oct4 could prevent the differentiation of ES cell lineages by acting like a co-repressor of lineage-specific transcription factors like FoxD3. Silencing of tau interferon genes

(IFN $\tau$ ) appears to be mediated by Oct4. IFN $\tau$  is expressed exclusively in the TE of bovine embryos and activated through the Ets-2 binding enhancer. Ezashi et al reported that Oct4 and Ets-2 could form a complex through interaction between the Oct4 POU domain and the DNA binding domain of Ets-2, and as a result quench the transactivation function of Ets-2. In trophoectodermal cells, Oct4 is down-regulated, thus, alleviating the co-repression of Ets-2 to allow the TE specific genes such as IFN $\tau$  to be expressed. These findings provided evidence that the developmental switch could be accomplished by the loss of Oct4 mediated silencing of key genes [16-19].



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**Fig.6.4 The phenotypic effects of gene-targeting experiments in mice.**

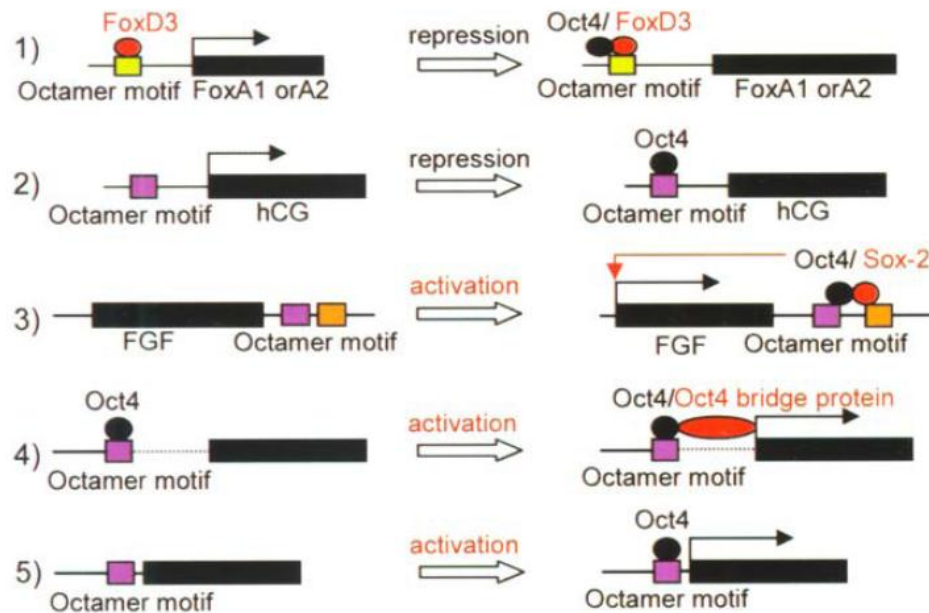
The figure depicts phenotypic effects of gene-targeting experiments in mice that provided insights into the pluripotency of embryos and embryonic stem cells. *Oct4*<sup>-/-</sup> (octamer-binding transcription factor-4), *Nanog*<sup>-/-</sup>, *Stat3*<sup>-/-</sup> (signal transducer and activator of transcription-3), *gp130*<sup>-/-</sup> (glycoprotein-130), *Lif*<sup>-/-</sup> (leukaemia inhibitory factor) and *Lifr*<sup>-/-</sup> (LIF receptor) embryos can develop to various stages *in vivo*— as highlighted by the outer ring in the figure, but they all fail (truncated line from the ring to the centre) to yield ESCs (embryonic stem cells) *in vitro* (see centre of the figure). So, ESCs need factors that

neither the ICM (inner cell mass) nor the primitive ectoderm require. It is important to make clear that ICM and ESCs might be equivalent but are not equal. ESCs might only represent some aspects of the natural embryo, and studying cells and phenomena *in vivo* is indispensable. E1.5, E3.5, E8.5 and E18.5 represent the number of days of embryonic (E) development, although developmental stages following E10.5 should properly be referred to as fetal.

A direct mechanism by which Oct4 can exert regulatory function involves trans-activation of target genes in stem cells. Oct4 can trans-activate its targets either proximally or remotely, depending on the location of its binding sites on the target promoters. Acting over a long distance, Oct4 may enlist the assistance of stem cell-specific co-activators that can bridge a remotely bound Oct4 protein to the basal transcription machinery. Quite unexpectedly, the adenovirus (Ad) E1A oncoprotein was found to be able to mimic the function of such stem cell-specific co-activators. Another oncoprotein, HPV-E7, was also reported to have a similar role in Oct4-mediated gene activation. Both proteins function as the bridging factors connecting a remotely bound Oct4 molecule to the general transcription machinery. Oct4 can also synergize with other transcriptional factors bound to the nearby cis-acting elements of target promoters. One such example concerns the regulation of FGF4 expression. FGF4 is a stem cell-specific growth factor, and has an enhancer element located within the 3'-untranslated region (UTR) of the gene, which is responsible for its stem cell-specific expression. This enhancer contains an octamer motif adjacent to a binding site to which Oct4 and the high mobility group (HMG) transcription factor Sox-2 bind cooperatively to activate transcription synergistically. This synergism is most likely mediated by protein-protein interactions. In the absence of Sox-2, Oct4 is not sufficient for FGF4 enhancer activity, even in the presence of the bridging factor E1A. In addition, the formation of Oct4/Sox-2 complex also appears to be a reciprocal event, since the complex could unmask latent activation domains in both proteins, thus leading to transcriptional activation. Interestingly, like the regulatory elements in FGF4 enhancer, the Sox-2 enhancer also has an octamer motif that can be regulated by Oct4/Sox-2



synergistically. The stem cell-specific gene *Utf1* is regulated through synergistic action of Oct4/Sox-2. These observations illustrate the versatility of Oct4, acting either as a suppressor of genes responsible for ES cell differentiation or as a trans-activator of genes known to retain the pluripotency of ES cells. As such, Oct4 can be considered as the primary factor that determines the fate of ES cells between self-renewal and differentiation [20-22].



Courtesy: Pan G.J et al., Cell Research (2002); 12(5-6):321-329

### **Fig.6.5 Modes of action of Oct4 on different target genes**

Oct4 represses gene expression either indirectly by neutralizing activators such as FOXD3 (example 1), or directly by binding to promoters (example 2). Oct4 also acts as an activator of gene transcription by binding to octamer sites located upstream (example 4 and 5) or downstream (example 3) of target genes. In the simplest mode, Oct4 binds to octamer sites immediately upstream of the promoter to activate gene expression directly (example 5). Alternatively, Oct4 can synergize with other factors like Sox2 to activate gene transcription (example 3). When located at a considerable distance, as in example 4, adaptor proteins must be involved to bridge Oct4 to the basic transcription machinery for transcriptional activation.

### ***Regulation and Functions of Oct-4 in Breast Cancer Cells***

Although much is known in terms of Oct-4 expression and functions in embryonic stem (ES) cells, information in breast cancer model systems is rather scarce. This is where the importance of our work stems as it highlights the crosstalk between Oct-3/4, a factor known to be indispensable for the pluripotency of ES cells, with ER $\alpha$  and Skp2, known to play a major role in proliferation in the breast. Our first cue for the involvement of Oct3/4 in ER $\alpha$  actions came upon our analysis of ChIP-seq datasets for mapping the genome-wide ER $\alpha$  binding sites, in MCF-7 breast cancer cells, in the presence of estradiol or tamoxifen (Brown 2007, Stunnenberg 2009) where we observed Oct3/4 and Nkx3 transcription factor binding sites enriched in the promoters of target genes that recruit ER $\alpha$  exclusively or preferentially in the presence of tamoxifen when compared to estradiol, a phenomenon validated later by ChIP analysis of putative targets. Interestingly, we found the expression of Oct-3/4 to be induced by tamoxifen, not estradiol, both at the level of transcript and protein in breast cancer cells. Hence, much of our work focused on investigating the mechanism underlying this tamoxifen specific upregulation of Oct-3/4 and the consequence of this on ER $\alpha$  target gene expression in response to tamoxifen.

## **EXPERIMENTAL PROCEDURES**

### ***Antibodies-***

Anti-Skp2 (N-19, H-435, A-2), anti-ER $\alpha$  (HC-20, F-10), anti-Ub (P4D1), anti-HA tag (F-7, Y-11), anti-Oct-1, 2, 3/4 and 11 were purchased from Santa Cruz Biotechnology. Anti-Myc tag (2272, 2276), anti-p38MAPK (9212, 9228), anti-phospho (Thr180/Tyr182) p38MAPK (9211, 9216) were from Cell Signaling and anti-flag rabbit and mouse and anti-phosphoserine antibody conjugated agarose beads were purchased from Sigma. Horseradish peroxidase (HRP)-conjugated donkey anti-goat, donkey anti-mouse and donkey anti-rabbit IgG secondary antibodies were from Santa Cruz Biotechnology.

### ***Cell Culture and Transfections-***

Cos-1 cells were purchased from American Type Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) [Hyclone] supplemented with 10% Fetal Bovine Serum (FBS) [Hyclone, Logan, UT], 100 units per ml of Penicillin and 50 µg/ml of Streptomycin at 37°C in 5% CO<sub>2</sub> with humidified air. MCF-7 cells were maintained in growth medium containing Minimum Essential Medium (MEM)-with phenol red (SIGMA) supplemented with 5% heat inactivated Calf Serum (CS) [Hyclone, Logan, UT], 100 units per ml of Penicillin and 50 µg/ml of Streptomycin at 37°C in non-CO<sub>2</sub> incubator or treatment medium containing Minimum Essential Medium (MEM)-without phenol red supplemented with 5% Charcoal dextran stripped Calf Serum (CS) at 37°C under 5%CO<sub>2</sub> in humidified air. All transient plasmid transfections were performed using LipofectAMINETM 2000 Reagent from Invitrogen according to manufacturer's instructions. siSkp2 transfection was performed using Lipitoid reagent kindly provided by Dr. Ronald Zuckermann at the Molecular Foundry, Lawrence Berkeley National Laboratory. All other siRNA transfections were performed using Dharmafect transfection reagent from Dharmacon.

### ***Plasmids-***

Skp2 cDNA was purchased by Open Biosystems and cloned into pcDNA3-Flag expression vector. Various Skp2 deletion mutants were generated from this full-length construct by PCR based subcloning. ERα was cloned into either pcDNA3-Flag expression vector or pcDNA3.1-Myc/His expression vector from Invitrogen. Site directed mutagenesis kit from Stratagene was used to introduce various Ser to Ala or Glu point mutations in pcDNA3-Flag-Skp2 (full-length). pCMV-HA-Ubiquitin was generated by subcloning from GST-Ubiquitin expression vector (Addgene plasmid 10861) originally generated by Peter Howley. pMEV-2HA-p38MAPK-WT (wildtype) and DN (dominant negative) mutant were purchased from Biomyx Technology. Adenoviral vectors encoding Skp2-WT or ΔF-box mutant were constructed by cloning

relevant sequences into pAdTrack vector purchased from Stratagene. Details for generation of any of the above mentioned expression vector, is available upon request.

#### ***Silencing by Small Interfering RNA (siRNA)-***

MCF-7 cells seeded onto 6-well plates at a confluence of 40-60% were transfected with siRNA duplexes, siSkp2 (Qiagen), sip38MAPK (Dharmacon), siNkx3-1 (Dharmacon), Lentiviral siOct-1 (Santa Cruz), Lentiviral siOct-2 (Santa Cruz), Lentiviral siOct-3/4 (Santa Cruz), Lentiviral siOct-11 (Santa Cruz), or non-targeting control siGL-3 (Dharmacon or Qiagen) and Lentiviral siGL-3 (Santa Cruz). siSkp2 transfection was performed using Lipitoid reagent kindly provided by Dr. Ronald Zuckermann at the Molecular Foundry, Lawrence Berkeley National Laboratory. All other siRNA transfections were performed using Dharmafect transfection reagent from Dharmacon.

#### ***Adenovirus Construction and Infection for Over-expression of Endogenous Factors-***

cDNAs for the genes of interest, namely Skp2-WT, Skp2 $\Delta$ Fbox, p38MAPK-WT, p38MAPK-DN (dominant negative), ER $\alpha$ , Oct-1, Oct-3/4, Oct-11, were first cloned into pAdTrack-CMV shuttle vector from Stratagene or pcDNA3.1-CMV vector using PCR based subcloning. DNA from positive clones of pAdTrack-CMV constructs was linearized by digestion with PmeI and electroporated into electrocompetent BJ5183 cells (Stratagene) already containing plasmid-P1 for the recombination event to occur. Recombined DNA was thereafter extracted and transfected into AD293 cells for adenovirus preparation. After rounds of amplification the virus obtained was then purified by CsCl density gradient centrifugation and characterized for its titre and ability to over-express the protein of interest. MCF-7 cells seeded onto 6-well plates at a confluence of 40-60% were thereafter infected with this virus or an equal MOI (Multiplicity of Infection) of a non target control virus followed by ligand treatment (if required). Cells were harvested and processed for RNA and/or protein extraction as per requirement. In some experiments an alternative strategy (Weigel et al.) was used to introduce genes of interest cloned in

regular pCMV-vectors into MCF-7 cells along with a poly-lysine coated adenovirus that was able to over-express the protein(s) of interest, namely Nkx3-1, NFATC4, by at least 2-3 folds.

#### ***Luciferase Reporter Gene Assay-***

MCF-7 or Hec-1 cells were transfected with plasmids of interest using LipofectAMINE 2000 following manufacturer's protocol. The dual luciferase assay system used was purchased from Promega Corp. (Madison, WI). Promoters for Oct-3/4 and NFATC4 genes were cloned into 2(ERE)-pS2-Luciferase gene-pCMV plasmid upstream of the pS2 gene and transfected along with Renilla luciferase gene plasmid (Clontech, Palo Alto, CA). Site-directed mutagenesis was performed to abrogate Nkx and Oct transcription factor binding sites in Oct-3/4 and NFATC4 promoters respectively using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. The mutagenesis sense and antisense primers were designed using the Stratagene web site. The plasmids were then sequenced to confirm the mutation of the desired site. Luciferase values were normalized using renilla luciferase gene as internal control for transfection efficiency. The amounts of plasmids transfected were: 1µg of the promoter constructs, 50 ng of Renilla luciferase construct, and 100 ng of ERα construct. Every experiment was performed at least three independent times.

#### ***Gene expression analysis by Quantitative Real-time PCR-***

MCF-7 cells seeded onto 6-well plates at a confluence of 40-60% were either siRNA transfected to silence the gene of interest or infected with adenovirus to over-express the gene of interest followed by treatment with ligand if necessary. RNA was isolated using Trizol reagent (Invitrogen) following manufacturer's protocol. The mRNA for genes of interest, namely Oct-1, Oct-2, Oct-3/4, Oct-11, Nkx3-1, NFATC4 was quantitated by SYBr Green-based reverse transcriptase-PCR (RT-PCR) using the ABI7600ht Sequence Detection System (Applied Biosciences). All mRNA quantities were normalized against 36B4 and primer sequences are available upon request.

### ***Chromatin Immunoprecipitation (ChIP) and sequential ChIP (reChIP) assays-***

Chromatin Immunoprecipitation assays (ChIPs) were performed with minor modifications as described in Metivier et al. (Gannon 2003). MCF-7 cells were weaned in minimum essential medium (MEM) supplemented with 5% Charcoal Dextran (CD)-stripped calf serum for 4 days followed by treatment with vehicle (ethanol), E2 (10nM) or Tamoxifen (1 $\mu$ M) for 45', 4h or 24h. In some experiments this ligand treatment was preceded by 48h long infection with Adeno-Skp2-WT or Adeno-Skp2 $\Delta$ Fbox, Adeno-p38MAPK-WT or Adeno-p38MAPK-DN, or equal MOI of a control adenovirus. Antibodies used for pull-downs were purchased from Santa Cruz Biotechnology [ER $\alpha$  (HC-20), Skp2 (N-19), Nkx3-1 (N-15), RNA polymerase II (N-20), Oct-3/4 (C-10 and N-19)], Abcam [RNA polymerase II (ab817), RNA polymerase II phospho-Ser2 (ab5095), RNA polymerase II phospho-Ser5 (ab5131)] and Bethyl Laboratories Inc. [p38MAPK (A310-212A)]. The DNA isolated was subjected to quantitative real-time PCR using gene specific primers (sequences available upon request) while using 36B4 as internal control (and measuring total input DNA in every sample) and a recruitment index was calculated (ratio between specific antibody signal over IgG signal). ChIP/reChIP experiments were done following the same ChIP protocol. After the first pull-down, immunoprecipitated material was recovered with 10mM dithiothreitol in IP buffer at 37°C for 30min, diluted and subjected to a second round of immuno-precipitation.

### ***Co-Immunoprecipitation assay-***

Cos-1 cells were seeded onto 6-well plates at a confluence of 80-90% and transfected with plasmids of interest. 24h post transfection cells were harvested, washed with ice-cold PBS and solubilized with 0.5ml CoIP buffer (50mM Tris pH7.5, 150mM NaCl, 0.1% Nonidet P-40, 5mM EDTA, 0.1mM sodium orthovanadate, 1mM dithiothreitol (DTT), 1mM PMSF and 1X Protease inhibitor cocktail from Roche). Immunoprecipitation was carried out using 2-5 $\mu$ g specific antibody by incubation at 4°C overnight with gentle rotation. The precipitated proteins were collected by incubating with Protein-G sepharose (GE

Healthcare) for 2h at 4°C with gentle rotation. After low speed centrifugation to remove the supernatant, the agarose was washed with CoIP buffer, boiled in SDS sample buffer and subjected to SDS-PAGE and Western blotting. In case of MCF-7 cells, endogenous proteins were Immunoprecipitated from lysates obtained from 10cm<sup>2</sup> plates using similar protocol as mentioned above with or without the involvement of transfections or adenoviral infections for exogenous over-expression of factors.

### ***Cell Proliferation assay-***

WST-1 cell proliferation assay is a colorimetric assay based on the cleavage of a tetrazolium salt, MTS, by mitochondrial dehydrogenases to form formazan in viable cells following the addition of WST-1, which can be detected by measuring absorbance at 450nm, which in turn is used as a measure of cell proliferation and viability. MCF-7-tamr cells were counted using a hemocytometer and seeded at 50,000 cells/well in a 12-well plate. Seeded cells were incubated overnight at 37°C with 5% CO<sub>2</sub> in a humidified atmosphere and next day treated with ligand, vehicle (ethanol), E2 (1nM) or Tamoxifen (1µM) and harvested after 0, 2, 4 and 6 days of treatment. At the time of harvest media was aspirated and 500µl of WST-1 reagent diluted 1:10 with 1XMEM supplemented with 5% CD stripped-Calf serum was added to each well. Incubation was continued for an additional 30min at 37°C with 5% CO<sub>2</sub> in a humidified atmosphere before absorbance was measured at 450nm versus a 650nm reference to compare the experimental samples to control, in Spectra MAX 190 microplate reader with SoftMax Pro software from Molecular Devices. Results were analyzed by exporting the data to MS Excel and expressing the mean ± SD for each sample, while every sample was run in triplicate.

## RESULTS AND EXPERIMENTAL FINDINGS

### ***Oct-3/4 Regulates Tamoxifen Driven Expression of NFATC4, an ER $\alpha$ Target Gene***

NFATC4 was found as one of the candidate genes, from the genome wide ChIP-seq dataset for ER $\alpha$  that recruited ER $\alpha$  preferentially in the presence of tamoxifen over estradiol, to its promoter that also had an Oct-transcription factor motif (Figure 6.6a). For gene expression analysis MCF-7 cells were transfected with siRNA against GL-3 (control) or Oct-1, 2, 3/4 or 11 followed by treatment with estradiol or tamoxifen for 24h. Interestingly, NFATC4 transcript was induced specifically upon treatment with tamoxifen, not estradiol and this tamoxifen mediated upregulation of NFATC4 was completely blocked in the presence of siRNA against Oct-3/4 both at the RNA (Figure 6.6b) and protein level (Figure 6.6c) thereby suggesting an important role of Oct-3/4 in regulating tam-bound ER $\alpha$  target gene expression, in this case of NFATC4.

### ***Oct-3/4 Impacts on NFATC4 Expression by Facilitating ER $\alpha$ Recruitment Through Binding to Response Elements in the NFATC4 Promoter***

To further confirm the role of Oct-3/4 in regulating tamoxifen specific induction of NFATC4, we performed luciferase assay in MCF-7 cells with WT or Oct-mut NFATC4 promoter, with abrogated Oct-3/4-binding site, fused to 2(ERE)-pS2-luciferase gene and found tamoxifen induction of luciferase gene driven by WT-NFATC4 promoter while a complete blockage of that in the presence of a mutant NFATC4 promoter (NFATC4-Luc-mutOct) with abrogated Oct-3/4-binding site (Figure 6.7a). On further investigation we found Oct-3/4 to be critical for the recruitment of ER $\alpha$  to target gene promoters, as we observed a significant loss in the occupancy of NFATC4 promoter by ER $\alpha$  in cells with reduced Oct-3/4 protein (Figure 6.7b).



### ***Oct-3/4 Impacts on Tamoxifen Sensitivity of Breast Cancer Cells by Regulating Tamoxifen Driven ER $\alpha$ Target Gene Expression***

To assess the role of p38MAPK initiated and Skp2 mediated Oct-3/4 induction and therefore elevated ER $\alpha$  target gene expression in the presence of tamoxifen, in regulating tamoxifen driven growth of breast cancer cells we compared the expression of Oct-3/4 and Nkx3-1 proteins between MCF-7 parental cells and MCF-7-tamr cells, their tamoxifen resistant derivatives. Interestingly, Oct-3/4 protein level was much elevated while Nkx3-1 protein notably reduced in MCF-7-tamr cells compared to MCF-7 parentals (Figure 6.8a). The Oct-3/4 modulated, tam-bound ER $\alpha$  target, NFATC4, a transcription factor known to enhance cytokine uptake by MCF-7 cells was found to induce the activation of the stress, cytokine induced p38MAPK in these cells, in a tamoxifen specific manner, thereby causing a positive feedback loop for tamoxifen driven Oct-3/4 induction leading to its own subsequent expression (Figure 6.8b).

To investigate the role of Oct-3/4 in acquired tamoxifen resistance, we performed growth assays in MCF-7-tamr cells with perturbed levels of Oct-3/4, by siRNA mediated knockdown (Figure 6.8c (i)) or adenovirus mediated over-expression (Figure 6.8c (ii)), following treatment with vehicle, estradiol or tamoxifen and found a significant impact of Oct-3/4 on the growth of cells in response to tamoxifen while only modest to no effects with estradiol. Also presence of ER $\alpha$  seemed critical for this regulation as the effect of Oct-3/4 on ligand induced growth of these cells was completely blocked in ER $\alpha$  negative, MDA-MB-468 cells (Figure 6.8d). Therefore, we conclude that Oct-3/4 which is induced by tamoxifen and has binding sites enriched in the promoters of tamoxifen bound ER $\alpha$  target genes regulates gene expression in breast cancer cells, largely by impacting the recruitment of tamoxifen bound ER $\alpha$  to target gene promoters thereby modulating tamoxifen sensitivity and tamoxifen induced growth of these cells.

### ***Skp2 Regulates ER $\alpha$ -Oct3/4 Driven Gene Expression in the Presence of Tamoxifen***

Adenovirus mediated over-expression of either Skp2 or p38MAPK further enhanced the tamoxifen, not estradiol, driven induction of NFATC4 transcripts and protein in MCF-7 cells, as measured by quantitative real-time analysis (Figure 6.9a) and western blot analysis (Figure 6.9b) respectively thereby establishing a role for both Skp2 and p38MAPK in regulating tamoxifen driven ER $\alpha$ -Oct-3/4 target gene (NFATC4) expression. To further investigate the role of Skp2 and p38MAPK in modulating Oct-3/4 regulated and tam-bound ER $\alpha$  driven target gene expression, we performed co-immunoprecipitation assay by transfecting Cos-1 cells with Myc-tagged Oct-1, 2, 3/4 or 11 along with ER $\alpha$ , Skp2 or p38MAPK to examine whether Oct-3/4 is in complex with ER $\alpha$ , Skp2 and/or p38MAPK. Immunoprecipitation with specific antibody against ER $\alpha$  was able to pull-down all the four Oct isoforms thereby suggesting ER $\alpha$  to be in complex with all the Oct isoforms (data not shown) while similar analysis proved Skp2 to be in complex with only Oct-3/4 and 11 while p38MAPK with Oct-3/4 alone. Therefore, Oct-3/4 was the only isoform that was in complex with all the three proteins of interest, namely ER $\alpha$ , Skp2 and p38MAPK (Figure 6.10a).

We next wanted to investigate the interaction of endogenous Oct-3/4 with ER $\alpha$ , Skp2 and p38MAPK in MCF-7 breast cancer cells and also assess the role of ligand on their interaction. To this end we used lysates from MCF-7 cells treated with vehicle, estradiol or tamoxifen and immunoprecipitated Oct-3/4 using a specific antibody subsequently western blotting for ER $\alpha$ , Skp2 and p38MAPK. Interestingly, endogenous ER $\alpha$ , Skp2 and p38MAPK proteins in MCF-7 cells also interacted with Oct-3/4, interestingly in a tamoxifen dependent manner (Figure 6.10b). Of note, these proteins were found to complex with Oct-3/4 upon tamoxifen treatment of these breast cancer cells only at later time-point (24h), which is also when target genes (NFATC4, in our work) are regulated by tamoxifen bound ER $\alpha$ , in these cells, as known previously in the literature but not understood why. Upon subsequent

investigation by ChIP for Oct-3/4 followed by reChIP for ER $\alpha$ , Skp2 or p38MAPK we found all these proteins to be in the same transcriptional complex on NFATC4 promoter (Figure 6.10c) thereby suggesting the crosstalk between ER $\alpha$ , Skp2, p38MAPK and Oct-3/4 to be key for tamoxifen specific regulation of NFATC4.

***Skp2 Regulates Tamoxifen Sensitivity of MCF-7-tamr cells Through its Impact on ER $\alpha$ -Oct3/4 Driven Gene Expression in the Presence of Tamoxifen***

To investigate the role of Skp2 in acquired tamoxifen resistance, we performed growth assays in MCF-7-tamr cells with perturbed levels of Skp2, by siRNA mediated knockdown (Figure 6.11a) or adenovirus mediated over-expression (Figure 6.11b), following treatment with vehicle, estradiol or tamoxifen and found a significant impact of Skp2 on the growth of cells in response to tamoxifen while only modest to no effects with estradiol. This impact on growth was largely due to the role of Skp2 in impacting tamoxifen sensitivity of these cells (Figure 6.11c and d). However, in cells that were depleted of Oct-3/4 protein, adenovirus over-expression of Skp2 was no more able to induce growth in response to tamoxifen (Figure 6.11e) and also presence of ER $\alpha$  seemed critical for this regulation as the effect of Skp2 on ligand induced growth was completely blocked in ER $\alpha$  negative, MDA-MB-468 cells (Figure 6.11f). Therefore, we conclude that p38MAPK initiated and Skp2 mediated induction of Oct-3/4 in response to tamoxifen, a transcription factor shown by our work to be critical in regulating gene expression through tamoxifen bound ER $\alpha$  in MCF-7 cells, impacts significantly on gene expression in response to tamoxifen thereby modulating tamoxifen sensitivity and tamoxifen induced growth of these cells.

## DISCUSSION

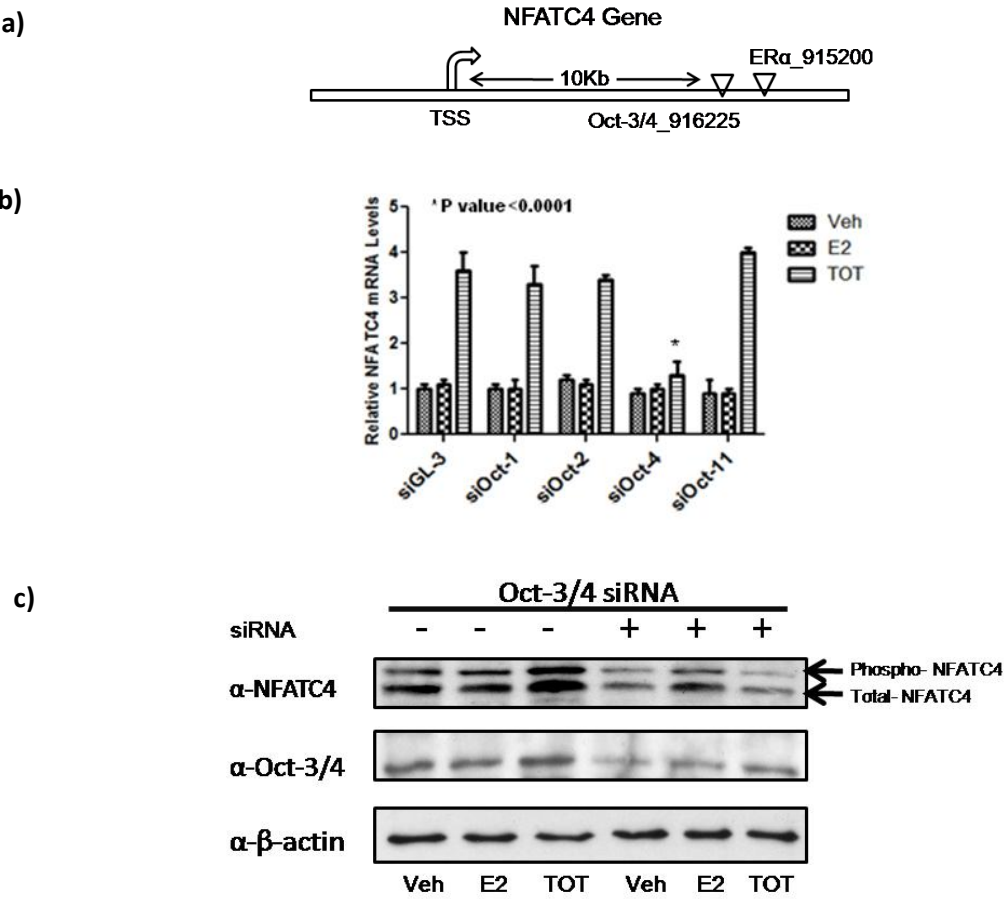
After insights into the mechanism underlying the tamoxifen specific induction of Oct-3/4 and its elevated expression in tamoxifen resistant MCF-7-tam<sup>r</sup> cells compared to parental cells we investigated the biological significance of the regulation if any in regulating tamoxifen sensitivity of these cells. Interestingly, perturbation of Oct-3/4 levels in these cells significantly impacted tamoxifen sensitivity and hence tamoxifen driven growth, the underlying mechanism for which needed to be determined. Since a lot of studies in embryonic stem cells, some on the genome-wide scale, point towards a crucial role of Oct-3/4 in regulating gene expression by direct recruitment to target gene promoters, describing this as an underlying mechanism for the regulation of pluripotency of these cells, it was tempting to speculate if Oct-3/4 could impact on ER $\alpha$  target gene expression in breast cancer cells. impact on tamoxifen sensitivity of breast cancer cells, we wanted to investigate the consequences and the likely impact of Oct-3/4 on ER $\alpha$  actions unique to tamoxifen. The biological significance of Oct-3/4 driven tamoxifen resistance was supported by the observation of elevated. Consistent with these findings, we were able to show significant impact of perturbation of Oct-3/4 levels on the ability of tamoxifen to promote growth of these cells, with little effect on estradiol driven growth.

At this point although we had insights into the pathway leading to the tamoxifen specific induction of Oct-3/4, the underlying mechanism for its role in mediating tamoxifen resistance in breast cancer cells was unclear. Further investigation into the mechanism by which Oct-3/4 impacts profoundly on the growth of breast cancer cells subjected to tamoxifen, identified Oct-3/4 to be a co-regulator of ER $\alpha$  target gene expression rather preferentially in response to tamoxifen, not estradiol. Our first cue for the involvement of Oct3/4 in ER $\alpha$  actions came upon our analysis of ChIP-seq datasets for mapping the genome-wide ER $\alpha$  binding sites, in MCF-7 breast cancer cells, in the presence of estradiol or tamoxifen (Stunnenberg 2009) where we observed Oct3/4 and Nkx3 transcription factor binding sites enriched in

the promoters of target genes that recruit ER $\alpha$  exclusively or preferentially in the presence of tamoxifen when compared to estradiol, a phenomenon validated later by ChIP analysis of putative targets. Our findings show a major impact of Oct-3/4 on the recruitment of tam-bound ER $\alpha$  to target gene promoters as loss of Oct-3/4 expression resulted in significantly dampened ER $\alpha$  recruitment. This suggests that Oct-3/4 upon induction with tamoxifen, gets recruited to the promoters of ER $\alpha$  target genes that recruit the nuclear receptor far more preferentially with tamoxifen, over estradiol, and while being their Oct-3/4 most likely facilitates ER $\alpha$  binding to these tamoxifen bound ER $\alpha$  target genes. One of the candidate tam-bound ER $\alpha$  target, modulated by direct recruitment of Oct-3/4 to its promoter was NFATC4 (Nuclear factor of activated T-cells, cytoplasmic 4). Oct-3/4 was found to complex with ER $\alpha$ , Skp2 and p38MAPK exclusively in the presence of tamoxifen, not estradiol, only at later time points (24h), which is also when most of the tamoxifen target genes are known to be regulated, NFATC4 being one of them as shown by our work. This highlights the importance and specificity of Oct-3/4 for gene regulation through tam-bound ER $\alpha$  even more, while explaining the absence of any effect in response to estradiol.

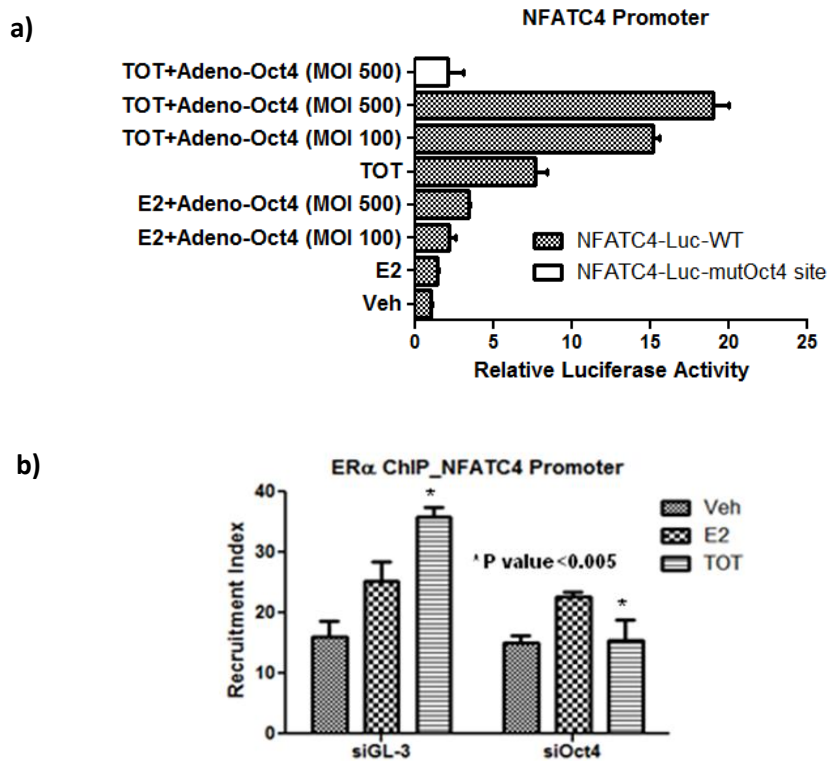
Our findings reveal Oct-3/4 mediated tamoxifen specific induction of NFATC4 through ER $\alpha$ , facilitated by Skp2 and p38MAPK, activating cytokine gene expression and therefore an influx of inflammatory cytokines (Rao A et al., 1997) to further activate p38MAPK thereby establishing a positive feedback loop for the induction of Oct-3/4 by tamoxifen, as a likely mechanism underlying the acquisition of tamoxifen resistance by breast cancer cells. Taken together our work documents a novel p38MAPK-Skp2-Oct-3/4 axis that impacts on gene regulation unique to tamoxifen bound ER $\alpha$ , thereby driving tamoxifen resistance in these cells, targeting of which we believe could circumvent or even reverse acquisition of tamoxifen resistance thereby making tam-resistant tumors amenable to endocrine therapy.

FIGURES



**Fig.6.6 Oct-3/4 Regulates Tamoxifen Driven Expression of NFATC4, an ERα Target Gene**

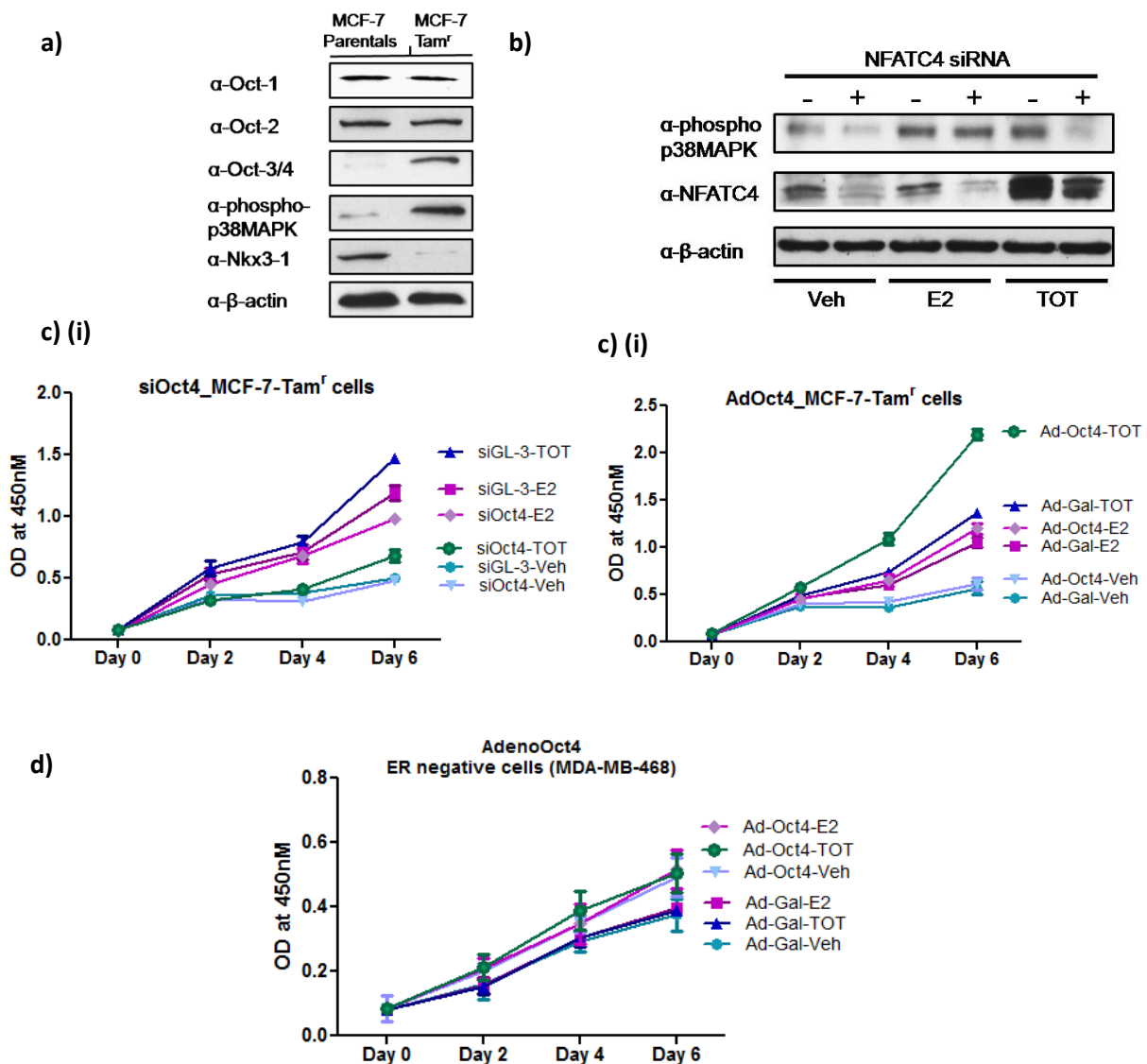
a) Bioinformatics analysis: Analysis of transcription factor binding sites in the NFATC4 promoter revealed a consensus site for Oct-3/4. b) Quantitative real-time analysis: Q-PCR analysis to examine the expression of NFATC4, using 36B4 as internal control, in MCF-7 cells transfected with either control non-targeting siRNA or siOct-3/4 and subsequently treated with vehicle (0.1% ethanol), E2 (10nM) or TOT (1μM) for 4 and 24h. c) Western blot analysis: Same experiment as described in (b) with 24h ligand treated samples processed for western blot analysis to examine NFATC4 protein.



**Fig.6.7 Oct-3/4 Impacts on NFATC4 Expression by Facilitating ERα Recruitment Through Direct Binding to Response Elements in the NFATC4 Promoter**

a) Luciferase Reporter Assay: MCF-7 cells were either transfected with siRNA against Oct-3/4 or a non-targeting control siRNA and 24h post transfection were transfected with either NFATC4-Luc-WT (wildtype NFATC4 promoter fused to 2ERE-pS2-Luciferase reporter) or NFATC4-Oct-3/4mut-Luc (NFATC4 promoter with abrogated Oct-binding site fused to 2ERE-pS2-Luciferase reporter) followed 6h post transfection by treatment with vehicle, E2 (10nM) or TOT (1μM) for 24h. Renilla luciferase was internal control for transfection efficiency. b) MCF-7 cells transfected with either specific siRNA against Oct-3/4 or a non-targeting control siRNA (siGL-3) followed 48h after by treatment with vehicle (0.1% ethanol), E2 (10nM) or TOT (1μM) for 24h were subjected to ChIP assays performed as described in materials and methods, using specific antibodies against ERα or IgG negative control. Data are averages ± standard

errors of the means of the results of three or more independent experiments and are represented as the recruitment index (specific antibody/IgG signal ratio).

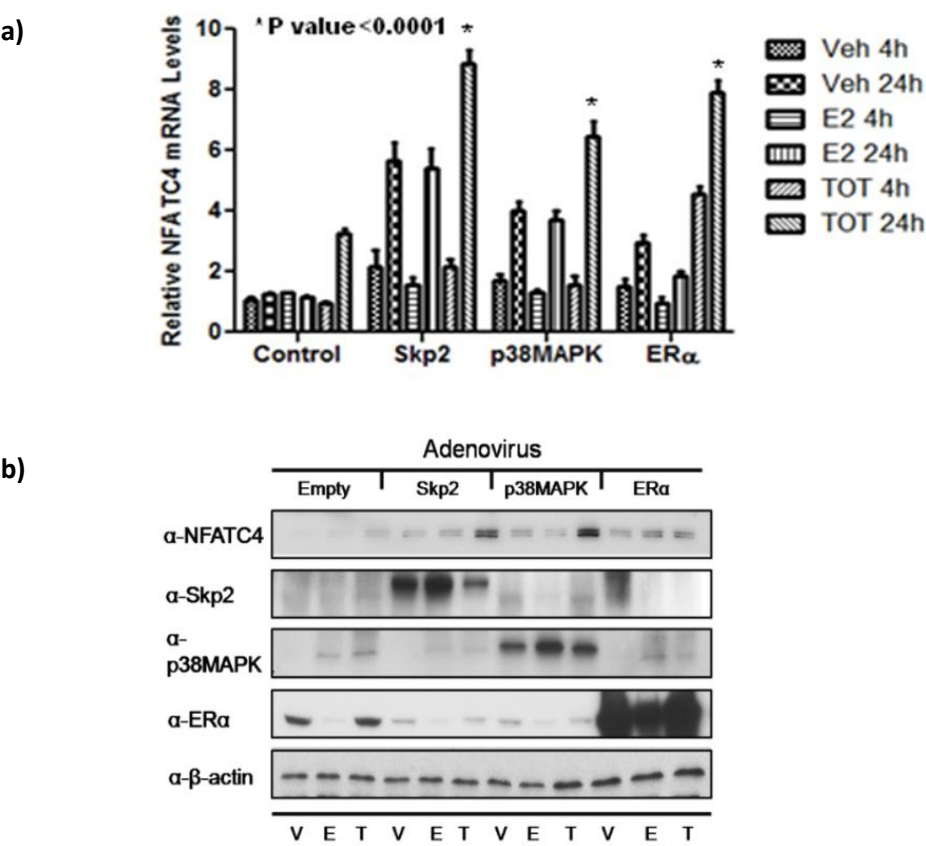


**Fig.6.8 Oct-3/4 Impacts on Tamoxifen Sensitivity of Breast Cancer Cells by Regulating Tamoxifen Driven ER $\alpha$  Target Gene Expression**

a) Western blot: Expression of Oct-1, 2, 3/4 and 11 along with Nkx3-1 and phospho-p38MAPK in MCF-7 parental cells vs MCF-7-tam<sup>r</sup>. b) Transfection of MCF-7 cells with either control siRNA or siNFATC4 and treatment with vehicle, E2 (10nM) or TOT (1 $\mu$ M) for 24h followed by harvest and protein analysis. c) (i)

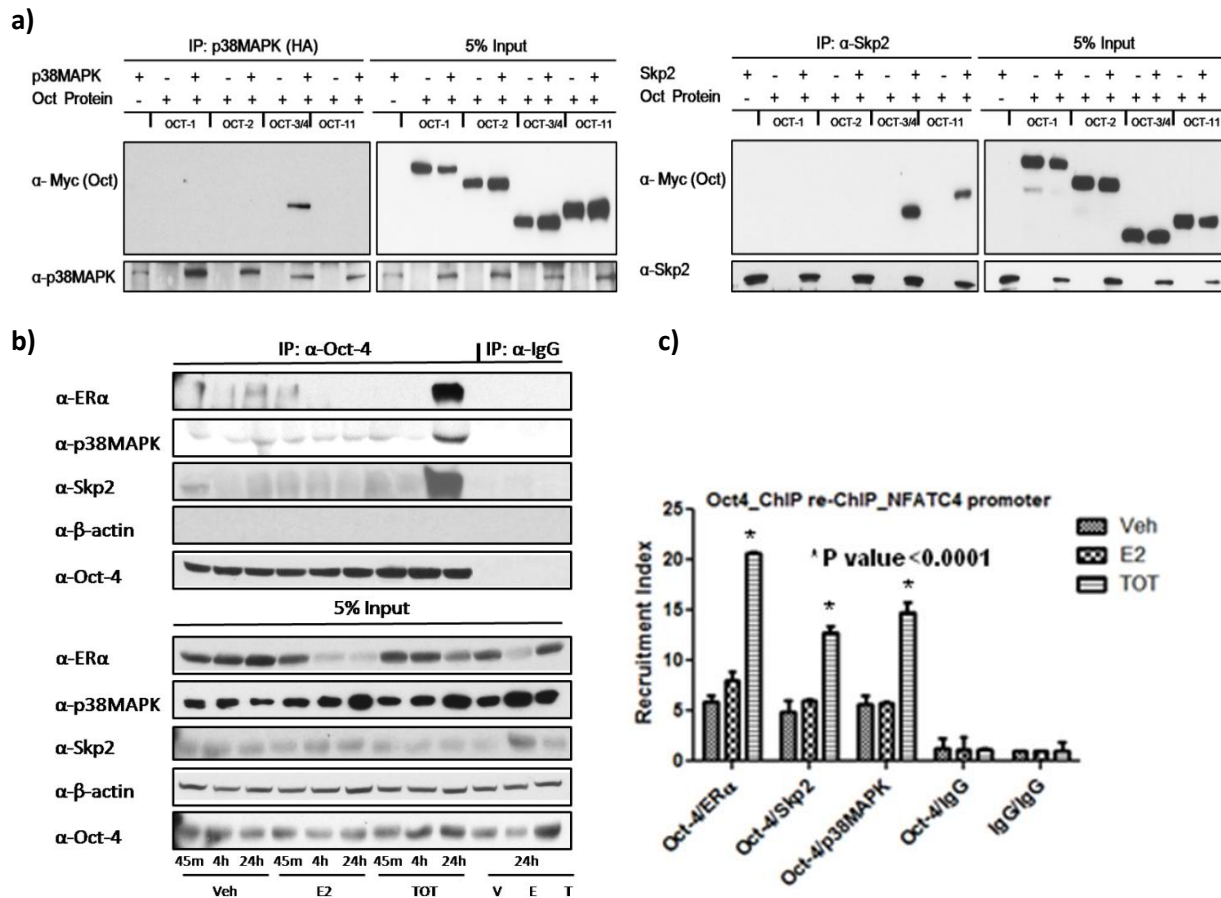


and (ii) Proliferation assays in MCF-7-tam<sup>r</sup> cells subjected to either siRNA knockdown or adenovirus over-expression of Oct-3/4 followed by treatment with vehicle, E2 (10nM) or TOT (1μM) for 0, 2, 4 and 6 days. d) Proliferation assay: Growth assay same as (c) in ER negative, MDA-MB-468 cells.



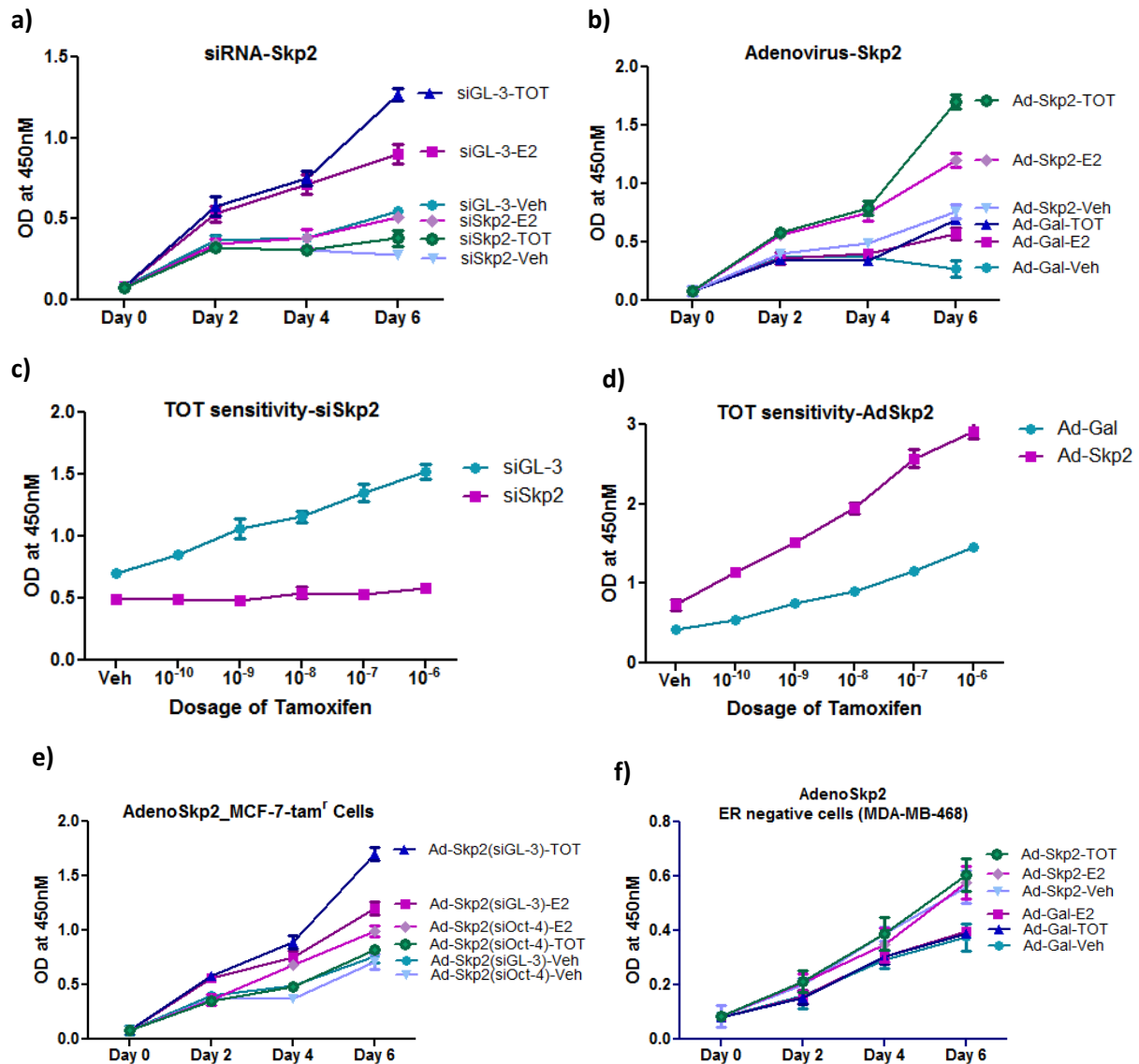
**Fig.6.9 Skp2 Regulates ERα-Oct3/4 Driven Gene Expression in the Presence of Tamoxifen**

a) Quantitative real-time analysis: Q-PCR analysis to examine the expression of NFATC4 in MCF-7 cells infected with Adeno-Control (β-Gal), Adeno-Skp2 or Adeno-p38MAPK for 48h including 4 and 24h treatment with vehicle (0.1% ethanol), E2 (10nM) or TOT (1μM), before harvest. 36B4 was used as an internal control. b) Western blot analysis: Same experiment as described in (a) with 24h ligand treatment was processed for western blot analysis to examine NFATC4 protein.



**Fig.6.10 Skp2, p38MAPK and ERα Complex with Oct-3/4 Specifically in the Presence of Tamoxifen**

a) CoIP: Cos-1 cells were transfected with either Myc-tagged Oct-1 or 2 or 3/4 or 11 alone or along with Flag-ERα (panel i), HA-p38MAPK (panel ii) or Flag-Skp2 (panel iii) and subjected to immunoprecipitation using anti-HA tag in (i) and anti-Skp2 in (ii) to pull-down p38MAPK and Skp2 resp. followed by WB with anti-Myc, p38MAPK and Skp2. b) CoIP: MCF-7 cells treated with vehicle, E2 or TOT for 45min, 4h and 24h were subjected to immunoprecipitation using anti-Oct-3/4 followed by western blotting with anti-Oct-3/4, anti-ERα, anti-p38MAPK and anti-Skp2 antibodies. e) ChIP re-ChIP: MCF-7 cells treated with vehicle, E2 (10nM) or TOT (1μM) for 24h were subjected to ChIP assays using specific antibodies against Oct-3/4 or IgG negative control. After the first pull-down, immunoprecipitated material was recovered with 10mM dithiothreitol in IP buffer at 37°C for 30min, diluted and subjected to a second round of immunoprecipitation using specific ERα, Skp2, p38MAPK antibodies or IgG negative control.



**Fig.6.11 Skp2 Regulates Tamoxifen Sensitivity of MCF-7-tam<sup>r</sup> cells through its Impact on ER $\alpha$ -Oct3/4 Driven Gene Expression in the Presence of Tamoxifen**

a, b) Same as fig.3c but with Skp2. c, d) Tamoxifen sensitivity assay in MCF-7-tam<sup>r</sup> cells with knockdown or over-expression of Skp2. e) Proliferation assay in MCF-7-tam<sup>r</sup> cells subjected to siRNA transfection with either siOct-3/4 or non-targeting siGL-3 followed 24h after by infection with Skp2 or control adenovirus and subsequent treatment with vehicle, E2 (10nM) or TOT (1 $\mu$ M) for 0, 2, 4 and 6 days.

Colorimetric assessment of cell density was performed using WST reagent. f) Proliferation assay in ER negative, MDA-MB-468 cells performed as in (b).

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## ABBREVIATIONS

ER $\alpha$	Estrogen Receptor- $\alpha$
ER $\beta$	Estrogen Receptor- $\beta$
PgR	Progesterone Receptor
GR	Glucocorticoid Receptor
E2	Estradiol
TOT	Tamoxifen
AF1 or 2	Activation Function-domain 1 or 2
DBD	DNA Binding Domain
EREs	Estrogen Response Elements
CoIP	Co-Immunoprecipitation Assay
ChIP	Chromatin Immunoprecipitation Assay
Skp2	S-phase Kinase-associated Protein 2
p38MAPK	p38 Mitogen Activated Protein Kinase
Cdk2	Cyclin Dependent Kinase 2
HA-Ub	HA-tagged Ubiquitin
NFATC4	Nuclear Factor of Activated T-cells Cytoplasmic calcineurin-dependent 4
UPS	Ubiquitin Proteasome System
ESCs	Embryonic Stem Cells
ICM	Inner Cell Mass
TE	Trophoectoderm

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**Bhatt S**, and Katzenellenbogen, B.S. Regulation of Estrogen Receptor Alpha (ER $\alpha$ ) Turnover by the Ubiquitin ligase, Skp2, in Breast Cancer Cells. [Ready for communication].

**Bhatt S**, and Katzenellenbogen, B.S. Interplay between Skp2, p38MAPK and Estrogen Receptor Alpha (ER $\alpha$ ) to drive Tamoxifen resistance in Breast Cancer Cells. [Ready for communication].

## Abstracts:

**Bhatt S.**, Stossi F., Katzenellenbogen BS. Regulation of Estrogen Receptor Alpha (ER $\alpha$ ) Turnover by the Ubiquitin ligase, Skp2, in Breast Cancer Cells. ENDO 2009 Annual Meeting. Washington DC, June 2009.

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**Bhatt S.**, and Katzenellenbogen, B.S. Regulation of Estrogen Receptor Alpha (ER $\alpha$ ) Turnover by the E3 Ubiquitin Ligase, Skp2, in Breast Cancer Cells. 22nd Annual Cell and Molecular Biology/ Molecular Biophysics Symposium, University of Illinois Urbana-Champaign, October 2009.

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